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(54) Improved vectors and methods for making such vectors and for expressing cloned genes.

(57) Improved vectors and methods for expressing cloned genes of prokaryotic or eukaryotic origin and methods of making such vectors are disclosed, the improved vectors comprising promoters and operators from λ phages and preferably do not include an active *cro* gene or an active *N* gene, the vectors having at least one endonuclease recognition site for cloning desired genes less than about 300 base pairs from the promoters and operators and being useful, as are methods utilizing the vectors, in producing a wide variety of prokaryotic, eukaryotic and viral polypeptides, hormones, enzymes, antigens, proteins and amino acids.

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IMPROVED VECTORS AND METHODS FOR MAKING SUCH VECTORS AND FOR EXPRESSING CLONED GENES

TECHNICAL FIELD OF INVENTION

This invention relates to improved vectors and methods for making such vectors and for expressing cloned genes. The vectors and methods disclosed herein are characterized by the improved expression of cloned genes particularly those of eukaryotic origin in prokaryotic hosts. As will be appreciated from the disclosure to follow, these vectors and methods may be used to improve the production of various polypeptides, proteins and amino acids in host cells.

BACKGROUND ART

The level of production of a protein in a host cell is governed by three major factors: the number of copies of its gene within the cell, the efficiency with which those gene copies are transcribed and the efficiency with which the resultant messenger RNA ("mRNA") is translated. Efficiency of transcription and translation (which together comprise expression) is in turn dependent upon the nucleotide sequences which are normally situated ahead of the desired coding sequence. These nucleotide sequences or expression control sequences define, inter alia, the location at which RNA polymerase interacts (the promoter sequence) to initiate transcription and at which ribosomes bind and interact with the mRNA (the product of transcription) to initiate translation.

Not all such expression control sequences function with equal efficiency. It is thus often of advantage to separate the specific coding sequence for a desired protein from its adjacent nucleotide sequences and to fuse it instead to other expression control sequences so as to favor higher levels of expression. This having been achieved, the newly engineered DNA fragment may be

inserted into a higher copy number plasmid or a bacteriophage derivative in order to increase the number of gene copies within the cell and thereby further to improve the yield of expressed protein.

Because over-production of even normally non-toxic gene products may be harmful to host cells and lead to decreased stability of particular host-vector systems, a good expression control sequence, in addition to improving the efficiency of transcription and translation of cloned genes, should also be controllable so as to modulate expression during bacterial growth. For example, the preferred expression control sequences are ones that may be switched off to enable the host cells to propagate without excessive build-up of gene products and then to be switched on to promote the expression of large amounts of the desired protein products.

Several expression control sequences, which satisfy some of the criteria set forth above, have been employed to improve the expression of proteins and polypeptides in bacterial hosts. These include, for example, the operator, promoter and ribosome binding and interaction sequences of the lactose operon of E. coli (e.g., K. Itakura et al., "Expression In Escherichia coli Of A Chemically Synthesized Gene For The Hormone Somatostatin", Science, 198, pp. 1056-63 (1977); D.V. Goeddel et al., "Expression In Escherichia coli Of Chemically Synthesized Genes For Human Insulin", Proc. Natl. Acad. Sci. USA, 76, pp. 106-10 (1979)), the corresponding sequences of the tryptophan synthetase system of E. coli (J.S. Emtage et al., "Influenza Antigenic Determinants Are Expressed From Haemagglutinin Genes Cloned In Escherichia coli", Nature, 283, pp. 171-74 (1980); J.A. Martial et al., "Human Growth Hormone: Complementary DNA Cloning And Expression In Bacteria", Science, 205, pp. 602-06 (1979)) and the major operator and promoter regions of phage λ (H. Bernard et al., "Construction Of Plasmid Cloning Vehicles That Promote Gene Expression From The Bacteriophage Lambda P_L Promoter", Gene, 5, pp. 59-76 (1979)). This invention relates to the last of

these expression control sequences.

Bacteriophage λ contains three major promoters -- P_L, P_R and P'_R. A repressor protein, the product of phage gene cI, is known to control the activity of promoters P_L and P_R. The repressor binds to the respective operator regions -- O_L and O_R -- of these promoters and blocks initiation of transcription from the corresponding promoter. Moreover, due to its autoregulatino mode of synthesis (M. Ptashne et al., "Autoregulation And Function Of A Repressor In Bacteriophage λ", Science, 194, pp. 156-61 (1976)), one copy of the cI gene on the chromosome of a lysogenic strain is able to repress fully the P_L or P_R promoters present in a multi-copy plasmid (infra). It should be noted that in systems involving the lac promoter repression of the promoter under non-induced conditions is only partial (K. Itakura et al., supra; D.V. Goeddel et al., supra).

The control exerted by the repressor over promoters P_L and P_R may be altered by modification of the repressor protein or its gene. For example, one mutation is known where the repressor protein is temperature sensitive. When that mutation is employed, the promoters may be activated or inactivated by varying the temperature of the culture and hence the stability of the repressor.

Bacteriophage λ also contains genes N and cro. The N gene is under P_L control. The product of the N gene is known to act as an anti-terminator in bacteriophage λ. Anti-termination is advantageous in overriding transcript termination or slow-down caused by the presence of termination sequences, termination-like sequences or transcription slow-down sequences in the particular DNA sequences that are to be transcribed. Furthermore, polarity effects, introduced by the presence of nonsense codons in the promoter transcript, may be relieved by the N gene product (N. Franklin & C. Yanofsky, "The N Protein Of λ: Evidence Bearing On Transcription Termination, Polarity And The Alteration Of E. coli RNA Polymerase", in RNA Polymerase (Cold Spring Harbor Laboratory) pp. 693-706 (1976)).

The product of the cro gene transcribed from the P_R promoter is known to be a secondary repressor for both promoters P_L and P_R (J. Pero, "Deletion Mapping Of The Site Of The tof Gene Product", in The Bacteriophage λ, (Cold Spring Harbor Laboratory), pp. 549-608 (1971); H. Echols, "Role Of The cro Gene In Bacteriophage λ Development", J. Mol. Biol., 80, pp. 203-16 (1973); A. Johnson et al., "Mechanism Of Action Of The cro Protein Of Bacteriophage λ", Proc. Natl. Acad. Sci. USA, 75, pp. 1783-87 (1978)). Because the cro gene product is co-produced along with the desired products of the host-vector combination, the cro gene product's effect on expression from the P_L or P_R promoters tends to increase with time. Therefore, in any system where continued high levels of expression are desired, deletion or inactivation of the cro gene is necessary.

The effectiveness of the P_L promoter for expression of cloned genes has been demonstrated by incorporating the tryptophan (trp) operon of E. coli into phage λ. (N. Franklin, "Altered Reading Of Genetic Signals Fused To The N Operon Of Bacteriophage λ: Genetic Evidence For Modification Of Polymerase By The Protein Product Of The N Gene", J. Mol. Biol., 89, pp. 33-48 (1979); A. Hopkins et al., "Characterization Of λ trp - Transducing Bacteriophages Made In Vitro", J. Mol. Biol., 107, pp. 549-69 (1976)). In this modified phage, the trp genes can be transcribed either from their own promoter or from the P_L promoter. P_L mediated expression was found to be 3-4 times higher than the levels obtained from the homologous trp promoter.

The effect of repressor on P_L mediated expression was also demonstrated in this modified phage. For example, in the absence of repressor, P_L controlled expression of antranilate synthetase (the first enzyme in the trp operon) was 11 times greater than that observed for the enzyme under trp promotion in the absence of trp repressor (J. Davison et al., "Quantitative Aspects Of Gene Expression In A λ trp Fusion Operon", Molec. gen. Genet., 130,

pp. 9-20 (1974)). Yet, in the presence of an active cI gene, P_L mediated expression of the enzyme was reduced at least 900-fold. These studies also demonstrated that continued high level of P_L mediated transcription was only possible if the cro gene was not functional in the host.

The problem is that although the above-described λ trp phages demonstrate the utility of the P_L promoter for the expression of inserted genes, the use of such phages is somewhat restricted by difficulties in construction and stable propagation of cro^- acceptor phages. Without such phages, the observed high levels of expression soon drop off as the level of the co-produced cro gene product increases and represses transcription from the P_L promoter.

While the disadvantage of λ phages has been somewhat overcome by cloning the λ control elements on an autonomously replicating plasmid such as Col EI or its derivatives (J. Hedgpeth et al., "Lambda Phage Promoter Used To Enhance Expression Of A Plasmid-Cloned Gene", Molec. gen. Genet., 163, pp. 197-203 (1978)) or by constructing smaller plasmids that incorporate only the λ P_L system (H. Bernard et al., supra), these latter vectors are disadvantaged by the distance between the sites available for insertion of cloned genes and the P_L promoter. For example, in the vectors described by H. Bernard et al., supra, the distance between the sites of gene insertion and the P_L promoter on the vector range from about 300 to about 8600 bases. Moreover, the more commonly used EcoRI and BamHI insertion sites in Bernard et al.'s vectors are not closer than 600 to 1000 bases, respectively, to the P_L promoter. In addition, the effect of the N gene product on transcription of the desired DNA sequences cannot be readily assessed in Bernard et al.'s vectors because the N gene product is encoded on the plasmid itself and is not of chromosomal origin. Finally, in addition to there being no direct evidence that Bernard's

vectors afford higher levels of protein expression, there is no teaching in Bernard that his vectors are usefully employed in the expression of eukaryotic gene products in prokaryotic hosts.

DISCLOSURE OF THE INVENTION

The present invention solves the problems referred to by providing an improved vector and method for making such vectors and for expressing cloned genes in host cells.

More specifically, we provide in accordance with this invention a vector comprising at least one DNA sequence comprising at least one promoter and operator derived from bacteriophage, characterized by at least one endonuclease recognition site located less than about 300 base pairs from that portion of said DNA sequence comprising said promoter and operator.

The major promoters of phage λ in the vectors of this invention promote the transcription of DNA sequences inserted into those vectors. The methods and vectors of this invention are further characterized by the presence of numerous appropriate recognition sites for the insertion of desired DNA sequences into the vectors near the chosen promoter. Preferably, the distance between the chosen promoter and the recognition sites is less than about 300 base pairs and more preferably less than about 150 base pairs. The preferred vectors of this invention are also those in which active N genes and active cro genes are absent. Therefore, by choice of appropriate host, i.e. one containing or lacking an active chromosomal N gene, any of the vectors of the invention may be employed for expression of DNA sequences in the presence or in the absence of the N gene product.

As will be appreciated from the description to follow, the vectors and methods of this invention permit the construction of host-vector combinations that enable

improved expression of prokaryotic and eukaryotic products in host cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic outline of a region of phage λ trp 44 cIAt$_2$ cro$^-$. Not all restriction sites have been depicted. The distances are mapped in λ units as described by E. Szybalski & W. Szybalski, "A Comprehensive Molecular Map Of Bacteriophage Lambda", Gene, 7, pp. 217-70 (1979).

Figure 2 is a schematic outline of the construction of vectors in accordance with this invention -- pPLa2, pPLa20 and pPLa23.

Figure 3 is a schematic outline of the construction of vectors in accordance with this invention -- pPLa2311, pPLa231 and pPLa8.

Figure 4 is a schematic outline of the construction of vectors in accordance with this invention -- pPLa83, pPLa831, pPLa832, pPLc2, pPLc23, pPLc236 and pPLc28.

Figure 5 is a schematic outline of the construction of vectors in accordance with this invention -- pPLc24.

Figure 6 displays the nucleotide sequence of the O_LP_L region of pPLa2311.

Figure 7 displays the conversion of a PstI site in β-lactamase to a BamHI site.

Figure 8 is an autoradiograph monitoring protein synthesis at 28°C and 42°C in E. coli K12ΔHI (pPLa23) and E. coli M5219 (pPLa23).

Figure 9 is an autoradiograph monitoring protein synthesis at 28°C and 42°C in E. coli K12ΔHI (pPLa23trpA$_1$) and E. coli K12ΔHI (pPLa23trpA$_2$).

Figure 10 is an autoradiograph monitoring protein synthesis at 28°C and 42°C in E. coli K12ΔHI (pPLc23trpA$_1$).

Figure 11 is an autoradiograph monitoring protein synthesis at 28°C and 42°C in E. coli K12ΔHI (pPLa2311R_1).

Figure 12 is a schematic outline of the construction of pPLc28SV$_t$5 and pPLc28SV$_t$5-37.

Figure 13 displays the construction of pPLc28SV$_t$5-37 from pPLc28SV$_t$5 on the nucleotide level.

Figure 14 is an autoradiograph monitoring the protein synthesis at 28°C and 42°C of E. coli K12ΔHI (pPLc28SV$_t$5-37-9) and the immunoprecipitation with serum from an SV40-tumor-bearing hamster of the proteins synthesized from this host after induction at 42°C as compared with immunoprecipitation of authentic small-t antigen synthesized in SV40-infected African green monkey kidney cells with the same antiserum.

BEST MODE OF CARRYING OUT THE INVENTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth.

In the description the following terms are employed:

Nucleotide - A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is called a nucleoside. The base characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C"), and thymine ("T"). The four RNA bases are A, G, C and uracil ("U").

DNA Sequence - A linear array of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Codon - A DNA sequence of three nucleotides (a triplet) which encodes, through its template or messenger RNA ("mRNA"), an amino acid, a translation start signal or a translation termination signal. For example, the

nucleotide triplets TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine ("Leu"), TAG, TAA and TGA are translation stop signals and ATG is a translation start signal.

Polypeptide - A linear array of amino acids connected one to the other by peptide bonds between the α-amino and carboxy groups of adjacent amino acids.

Structural Gene - A DNA sequence which encodes through its mRNA a sequence of amino acids characteristic of a specific polypeptide.

Transcription - The process of producing mRNA from a structural gene.

Translation - The process of producing a polypeptide from mRNA.

Expression - The process undergone by a structural gene to produce a polypeptide. It is a combination of transcription and translation.

Plasmid - A nonchromosomal, double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (Tet^R) transforms a cell previously sensitive to tetracycline into one which is resistant to it. A host cell transformed by a plasmid or vector is called a "transformant".

Phage or Bacteriophage - Bacterial virus many of which consist of DNA sequences encapsidated in a protein envelope or coat ("capsid").

Cloning Vehicle or Vector - A plasmid, phage DNA or other DNA sequence which is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition or restriction sites at which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological function of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites, and which contain a marker

suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance.

Cloning - The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

Recombinant DNA Molecule or Hybrid DNA - A molecule consisting of segments of DNA from different genomes (the entire DNA of a cell or virus) which have been joined end-to-end outside of living cells and have the capacity to infect some host cell and be maintained therein.

Expression Control Sequence - A sequence of nucleotides that controls and regulates expression of genes when operatively linked to those genes.

THE HOST CELLS OF THIS INVENTION

Any of a large number of available host cells may be used in the host-vector combinations of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art. These include, for example, compatibility with the chosen vector, toxicity of proteins encoded by the hybrid plasmid, ease of recovery of the desired protein, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all hosts may be equally effective for expression of a particular recombinant DNA molecule. Within these general guidelines, useful hosts may include strains of E. coli, Pseudomonas, Bacillus subtilis, Bacillus stearothermophilus, and other bacilli, yeasts and other fungi, animal or plant hosts such as animal (including human) or plant cells in culture or other hosts.

The preferred host cells of this invention are E. coli strains K12 $cI_{ts}\Delta HI$ (K12 M72 lac_{am} $\Delta trpEA2$ Sm^R ($\lambda cI857$ $N_{am7}N_{am53}\Delta HI$ bio^-)) ("K12ΔHI") (H. Bernard et. al., supra) and M5219 (K12 M72 lac_{am} trp_{am} Sm^R ($\lambda cI857$ ΔHI bio252)) ("M5219") (H. Greer, "The kil Gene Of Bacteriophage λ", Virology, 66, pp. 589-604 (1975)).

A. Vectors According To This Invention Containing The P_L Promoter In The Anti-Clockwise Orientation with Respect To The Origin Of Replication

1. pPLa23

Referring now to Figure 2, one improved vector of this invention pPLa23 was prepared in a sequence of steps. These are depicted in Figure 2 and more fully described below.

(a) Intermediate Plasmid pPLa2

The λ trp 44 $cIAt_2$ cro^- DNA isolated above was digested with BamHI and EcoRI to excise a fragment extending from about 71.3% to 81.02% on the λ map (Figures 1 and 2). In like manner, pBR322 was digested with BamHI and EcoRI and the phage λ DNA fragment inserted in place of the excised EcoRI-BamHI pBR322 fragment (Figure 2).

The resultant vector was designated pPLa2, the "a" serving to indicate the anticlockwise orientation of the P_L promoter with respect to the origin of replication. The λ information on this molecule extends from the BamHI site of the phage (71.3% λ) to the EcoRI site (81.02% λ) and includes the gene N, the O_LP_L region, genes rex and cI (mutant), the O_RP_R region, genes cro (mutant) and cII and part of gene O (Figures 1 and 2).

E. coli C600 ($CaCl_2$ competent) was transformed with the above prepared pPLa2 under appropriate conditions and containment. Transformants were selected at 34°C on LB plates seeded with 10^9 pfu of phage λ_{clear} mutant (M. Lieb, supra) and also containing 100 µg/ml carbenicillin. The chosen λ DNA fragment includes the $cIAt_2$ gene and therefore transformants containing this fragment will be resistant to phage λ_{clear} mutant at 34°C. In addition, the chosen pBR322 fragment includes the gene for ampicillin resistance so that hosts transformed with plasmids having that gene intact will grow in cultures containing that antibiotic to the exclusion of those hosts not so transformed.

Twenty transformants were selected and cultures grown at 34°C in LB medium containing 100 µg/ml carbenicillin and 10^{-2}M $MgCl_2$. To insure that the transformants were true transformants harboring a cI gene and not rare bacteria unable to adsorb λ phage, aliquots of the cultures were infected with either λ_{clear} or λ_{vir} (F. Jacob & E. Wollman, "Etude Génétique d'un Bacteriophage Tempere d'Escherichia Coli. I. Le Système Génétique du Bacteriophage λ", Ann. Inst. Pasteur, 87, pp. 653-90 (1954)). All twenty transformants displayed resistance to λ_{clear} and sensitivity to λ_{vir}.

Form I DNA from one of these twenty transformants was isolated using standard procedures, restricted with EcoRI and BamHI and sized against standard markers. The DNA displayed two bands corresponding to the expected sizes of the pBR322 fragment and the phage λ fragment.

(b) Intermediate Plasmid pPLa20 -- Elimination of BglII Fragments From pPLa2

The λ region of pPLa2 includes four BglII sites located at 73.77, 78.80, 80.16 and 80.28% λ (Figures 1 and 2) (V. Pirotta, "Two Restriction Endonucleases From Bacillus Globigi;", Nucleic Acids Res., 3, pp. 1747-60 (1976); H. Szybalski & W. Szybalski, "A Comprehensive Molecular Map of Bacteriophage λ", Gene, 7, pp. 217-70 (1979)).

To eliminate the BglII fragments between 73.77% λ and 80.28% λ, pPLa2 DNA was digested with BglII, religated at a DNA concentration of less than - µg/ml and transformed into E. coli W6 (λ_{rex}) ($CaCl_2$ competent) having a chromosomal λ repressor cI so as to silence P_L dependent transcription (Figure 2). Carbenicillin resistant clones were selected by growth in L-broth containing 100 µg/ml carbenicillin and screened for loss of λ_{rex} function using a T_4 rII 638 mutant. The λ_{rex} function prevents growth of the T_4 rII 638 mutant (B. Howard, "Phage λ Mutants Deficient In rII Exclusion", Science, 158,

pp. 1588-89 (1967)). Therefore, the failure of these BglII restricted transformants to prevent the growth of the T_4 rII 638 mutant as compared with the lack of growth of the mutant in hosts transformed with pPLa2 demonstrated that the rex function had been eliminated from the pPLa2 recombinant DNA molecule by the BglII deletion.

Restriction analysis of the recombinant DNA molecules of these BglII restricted transformants revealed the presence of a single BglII site. Moreover, digestion with EcoRI and BamHI produced two fragments -- one corresponding to the expected pBR322 fragment and the other to the expected size (1900 base pairs) of the phage λ DNA fragment after elimination of that portion between BglII sites 73.77% λ and 80.28% λ. This modified plasmid was designated pPLa20. Its λ DNA insert extends from the BamHI site (71.3%) to the BglII site (73.77%) and from the BglII site (80.28%) to the EcoRI site (81.02%). It includes gene N, the O_LP_L region and part of gene O (Figure 1).

While the remainder of this example of the construction of embodiments of vectors of this invention focuses on the P_L promoter -- the P_R promoter having been eliminated from pPLa2 with the BglII-BglII fragment -- it should be understood that similar manipulations could have been employed to eliminate the P_L promoter from pPla2 and to construct a vector retaining the P_R promoter. In addition, vectors within this invention could be constructed by similar means having both the P_L and P_R promoters present such that the two promoters act in concert or in opposition to mediate the expression of inserted DNA sequences.

(c) pPLa23 -- Introduction Of An EcoRI Site At A Short Distance Downstream From P_L

The BglII-BamHI fragment present on pPLa20 contains a single HaeIII site [73.1% λ, Figure 1] located about 150 nucleotides downstream from P_L (B. Allet and

R. Solem "Separation And Analysis Of Promoter Sites In Bacteriophage λ DNA By Specific Endonucleases" J. Mol. Biol. 85, 475-84 (1975)). This site can be converted into an EcoRI site by flush-end ligation of an open HaeIII end to an open EcoRI end previously flush-ended by extending the recessed 3'-end with DNA polymerase I in the presence of deoxyribonucleoside triphosphates (K. Backman et al. "Construction Of Plasmids Carrying The cI Gene Of Bacteriophage λ", Proc. Natl. Acad. Sci. U.S.A., 73, pp. 4174-78 (1976)). Details of the procedure are described below and illustrated in Figure 2.

Six pmol of pBR322 was digested with EcoRI. After heat-killing the enzyme, the DNA was precipitated and dissolved in 250 µl of a buffer containing 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 2 µM of each of the four deoxyribonucleoside triphosphates (with α-^{32}P-dATP (345 Ci ^{32}P/mmol)) and 50 µg BSA/ml. Six units of DNA polymerase I from E. coli (Worthington) were added and the mixture was incubated at 16°C for 90 min. This process resulted in the flush-ending of the open 3' EcoRI site in the linearized pBR322.

After heat-inactivation of the enzyme, the mixture was adjusted to 50 mM NaCl, 7 mM β-mercaptoethanol and the DNA digested with BamHI. The fragments were separated by electrophoresis on a 1.4% agarose gel and monitored by autoradiography. A gel slice containing the larger of the two fragments -- pBR322 containing an open BamHI site and a flush-ended EcoRI site -- was cut out and frozen at -90°C. This piece of agarose was then centrifuged (SS34 rotor (Sorvall)) for 20 min at 20,000 rpm. The expelled supernatant was removed and the freezing and centrifugation steps repeated two additional times. Under these conditions about 30% of the DNA contained within the agarose slice is expelled into the supernatant. The expelled DNA was precipitated from the combined supernatants and dissolved in 10 µl of 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 7 mM β-mercaptoethanol.

Two pmol of pPLa20 was digested with BglII and BamHI and the fragments separated on agarose gel. The smaller fragment (BglII-BamHI) was eluted from the gel as described above and digested with BspRI, an isoschizomer of HaeIII (A. Kiss et al. "A New Sequence-Specific Endonuclease (Bsp) From Bacillus Sphaericus", Gene, 1, pp. 323-29 (1977)) to produce a mixture of BamHI-BspRI and BspRI-BglII fragments, the latter carrying the P_L promoter. The enzymes BglII and BamHI make identical open ends such that an open BglII end can be ligated to an opened BamHI end and vice versa. Moreover, the result of either ligation is no longer a substrate for BglII or BamHI but is recognized by the enzyme Sau3AI (MboI) (V. Pirotta, supra).

Two pmol of the aforementioned pBR322-EcoRI-BamHI larger fragment was ligated to 0.8 pmol of the mixture of BamHI-BspRI and BspRI-BglII fragments. Following ligation (the open BamHI site on the pBR322 fragment is available for ligation to either the open BglII or BamHI sites of the pPLa20 fragments and the flush-ended EcoRI site of the pBR322 fragment is available for ligation to the BspRI (HaeIII) sites of the pPLa20 fragments), the mixture was digested with BamHI to eliminate those recombinant molecules comprising the unwanted BamHI-BspRI fragment inserted in the pBR322 vector. The resulting mixture was transformed into E. coli M5219 and transformants selected for resistance to carbenicillin. A total of 23 transformants was obtained. All of these transformants were sensitive to tetracycline (also carried by pBR322) because the BamHI restriction of pBR322 rendered the gene coding for Tet^R no longer intact in the modified plasmid (Figure 2).

The continued presence in these clones of the P_L-carrying BspRI-BglII fragment was checked by digesting the DNA with HincII. Since pBR322 contains two HincII sites (J. Sutcliffe, supra) and the expected λ P_L fragment contains a single HincII site (73.4% λ, Figure 1) (B. Allet and R. Solem, supra) correctly constructed recombinant

DNA molecules should contain three HincII sites. Of the 23 transformants obtained, five contained the three predicted HincII sites. Three of these had a unique EcoRI site indicating that in those clones the correct junction between the BspRI (HaeIII) site of the pPLa20 fragment and the flush-ended EcoRI site of the pBR322 fragment had been made. These three clones also lacked a BamHI site as predicted by the expected ligation of the BglII end of the pPLa20 fragment to the BamHI end of the pBR322 fragment. One of these clones was chosen for further work and was designated pPLa23 (Figure 2).

pPLa23 consists of a pBR322 fragment extending from the BamHI site (base pair 377 of pBR322) to the EcoRI site (base pair 4362 of pBR322) (J. Sutcliffe, supra) (Figure 2). The remaining part of pBR322 has been replaced in pPLa23 by the fragment of λ trp 44 $cIAt_2$ cro^- DNA located between the HaeIII site at 73.3% λ (now a reconstructed EcoRI site) and the BglII site at 73.77% λ (now an Sau3A site) (Figure 2). The size of this fragment was estimated by agarose gel electrophoresis to be about 300 base pairs. Within this fragment are contained the O_LP_L region and the first 115 nucleotides of the N gene transcript (J. Dahlberg & F. Blattner, supra). The direction of transcription of the P_L promoter is from the BglII site towards the HaeIII site and runs in the same sense as transcription from the β-lactamase promoter of pBR322 (J. Dahlberg & F. Blattner, supra; J. Sutcliffe, supra).

Two features of the plasmid are of special interest: 1) The regions coding for the P_L promoter and for the β-lactamase gene are present on a single HaeII fragment, delineated by the HaeII sites at base pair 2720 and 436 of pBR322 (Figure 3) (J. Sutcliffe, supra; B. Allet and R. Solem, supra; V. Pirotta, supra). 2) The origin of replication is located on a 370 base pair HaeII fragment adjacent to the β-lactamase-carrying HaeII fragment (Figure 3). A functional origin of replication requires

that the junction around the HaeII site at position 2720 be maintained (A. Oka et al. "Nucleotide Sequence Of Small Col El Derivatives. Structure Of The Regions Essential For Autonomous Replication And Colicin El Immunity", Mol. gen. Genet., 172, pp. 151-59 (1979)). These features of pPLa23 were utilized to introduce a second antibiotic resistance marker into the vector.

2. pPLa231 and pPLa2311 -- Introduction Of A Kanamycin Resistance Marker Into pPLa23

Referring now to Figure 3, the steps employed to prepare other vectors of this invention from pPLa23 are depicted. These steps are more fully described below.

A HaeII fragment coding for resistance to kanamycin was obtained from plasmid pMK20 (M. Kahn et al., "Plasmid Cloning Vehicles Derived From Plasmids ColEl, F, R6K and RK2", Methods in Enzymology, 68, pp. 268-80 (1979)). The origin of replication on plasmid pMK20 is largely contained within a 359 base pair HaeII fragment. However, the origin also spans the junction between this fragment and an adjacent HaeII fragment (M. Kahn et al., supra). The nucleotide sequence around this HaeII site is identical to the sequence found in pBR322 around the HaeII site at position 2720 (A. Oka et al., supra; J. Sutcliffe, supra).

A mixture of pPLa23 and pMK20 was digested to completion with HaeII, religated and transformed into E. coli M5219 ($CaCl_2$ competent) (Figure 3). Correctly transformed colonies were selected on the basis of their resistance to carbencillin and kanamycin, because only clones containing the β-lactamase gene from pBR322 and the kanamycin gene from pMK20 will display dual antibiotic resistance. Twelve dual resistant transformants were selected. Plasmid DNA was isolated from these transformants, as before, and analyzed by HaeII restriction and

fragment sizing on a 6% acrylamide gel. Five of these clones had only three HaeII fragments -- an HaeII fragment corresponding to the HaeII fragment of pPLa23 which carries the P_L promoter and β-lactamase gene, an HaeII fragment corresponding to the HaeII fragment of pMK20 carrying the gene for kanamycin resistance and a small HaeII fragment also derived from pMK20 and required for plasmid replication (Figure 3).

The five selected clones were further examined to determine the orientation of the kanamycin gene containing HaeII fragment from pMK20 with respect to the reconstructed EcoRI site in the HaeII fragment from pPLa23. The kanamycin gene containing HaeII fragment from pMK20 is known to contain a unique asymmetric HindIII site (M. Kahn et al., supra) (Figure 3). Therefore, this site provides a means of determining the orientation of the fragment.

The five clones were digested with HindIII and EcoRI and the resulting fragments sized as before. Four of the five clones had the larger portion of the HindIII-cleaved kanamycin gene containing HaeII fragment from pMK20 adjacent to the reconstructed EcoRI site. One clone had the opposite orientation. These two sets of clones were arbitrarily designated pPLa231 and pPLa2311, respectively (Figure 3).

pPLa2311 was arbitrarily selected from the above-constructed plasmids and the nucleotide sequence of the P_L region determined.

Preliminary to the sequencing, two sets of restriction fragments were prepared from pPLa2311 -- EcoRI-HincII fragments and HincII-EcoRI-XhoI fragments (not shown in Figure 3). In both cases pPLa2311 was digested with the first restriction enzyme and the resulting fragments labelled with ^{32}P using T_4 polynucleotide Kinase (P-L Biochemicals). Then, the fragments were digested with the second restriction enzyme or pair of enzymes, in the case of EcoRI-XhoI, and the fragments

separated on a 6% agarose gel. Sequencing was done conventionally using the procedures of A. Maxam & W. Gilbert, "A New Method For Sequencing DNA", Proc. Natl. Acad. Sci. USA, 74, pp. 560-64 (1977).

The nucleotide sequence of this region is displayed in Figure 6. It extends from the HaeII site in pBR322 to the reconstructed EcoRI site at the junction between the λ phage fragment and pBR322. The determined sequence has the following characteristics as compared to known sequences: (1) the nucleotide sequence of the O_LP_L operator-promoter region is identical to that sequence of this region in phage λ (T. Maniatis et al., supra); (2) the sequence between the HaeII site and the Sau3A site at the junction between the λ phage fragment and pBR322 is identical to that of authentic pBR322 (J. Sutcliffe, supra); (3) the sequence of the N gene transcript agrees with the sequence determined at the mRNA level by Dahlberg & Greenblatt (supra) except for a deletion of one adenosine residue at position 41 of the transcript; and (4) the sequence does not include the translational start signal of the N gene (N. Franklin & G. Bennett, "The N Protein Of Bacteriophage λ, Defined By Its DNA Sequence, Is Highly Basic", Gene, 8, pp. 107-19 (1979)).

3. pPLa4 and pPLa8 -- Conversion Of The PstI Site In The β-Lactamase Gene Of pPLa2311 To A BamHI Site

Figure 3 and 7 display in schematic outline the conversion of the PstI site in the β-lactamase gene of pPLa2311 to a BamHI site. Plasmid pPLa2311 was linearized with PstI. Following phenol and chloroform extraction, the DNA was precipitated, redissolved to a concentration of 50 pmol/ml in 25 mM $NaCOOCH_3$ (pH 4.5), 1 mM $ZnCOOCH_3$, 125 mM NaCl and treated with S1 nuclease (Sigma) at 1.5 units per pmol of DNA for 90 min at 25°C to remove the 3'-protruding ends (Figure 7). The reaction was terminated by addition of EDTA to 5 mM. S1 nuclease was

removed by incubating the mixture in the presence of 0.2% SDS for 10 min at 70°C followed by phenol and chloroform extraction. The DNA was precipitated by addition of 4 vol 2 M NH_4COOCH_3 and 14 vol ethanol.

The recovered DNA was blunt-end ligated to a 10-fold molar excess of BamHI linker molecules (Collaborative Research Inc.) (C. Bahl et al. "A General Method For Inserting Specific DNA Sequences Into Cloning Vehicles", Gene, 1, pp. 81-92 (1977)) (Figure 7). Following cleavage with BamHI and religation at low DNA concentration (Figure 7), the mixture was cleaved with PstI to counter-select those molecules that had escaped S1 nuclease treatment and retained an intact PstI site. Two µg of treated DNA was then transformed into E. coli M5219 and transformants selected by kanamycin resistance. A total of 10 transformants was obtained, two of which lacked a PstI site and had acquired a BamHI site. The recombinant DNA molecules of these latter two transformants were designated pPLa8 and pPLa4 (Figure 3, pPLa4 is not shown in Figure 3). The fragments obtained after combined EcoRI-BamHI digestion of the recombinant DNA molecules of these transformants comigrated on a 1.4% agarose gel with the fragments obtained from pPLa2311 after EcoRI-PstI cleavage. Therefore, the PstI site in pPLa2311 has been replaced by a BamHI site.

Referring again to Figure 7, the effect of the above-described sequence of steps on the β-lactamase gene is depicted. As illustrated in Figure 7, the final result of the construction is the replacement of the Ala amino acid residue at position 182 in the β-lactamase protein by the sequence Arg-Ile-Arg. Since this substitution will leave the reading frame of the β-lactamase gene intact, it was expected that transformants of the reconstructed clones would display resistance to carbencillin. Unexpectedly, host cells transformed with pPLa4 and pPLa8 were not resistant to carbencillin.

4. pPLa83 -- Introduction Of A BamHI Site Next To The EcoRI Site Of pPLa8

Plasmid pAD3 (a gift of H. Schaller) contains a 47 base pair sequence inserted into the BamHI site of pBR322. This sequence consists of the following units -- BamHI site-EcoRI site-lactose operator-EcoRI site-BamHI site. In order to insert this sequence into the reconstructed BamHI site of pPLa8, pPLa8 and a 10-fold excess of pAD3 were digested with BamHI, religated and transformed into E. coli W6 (λ_{rex}) (Figure 4). Transformants were selected on plates containing minimal medium, 50 µg/ml kanamycin, 0.1% glucose, 40 µg/ml X gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (J. Miller, Experiments In Molecular Genetics (Cold Spring Harbor Laboratory), p. 48 (1972)), because the presence of X gal dye allows detection of transformants which contain a lactose-operator fragment. In fact, in that medium lactose operator-containing transformants are blue and therefore easily distinguishable from other transformants.

The recombinant DNA molecules were isolated from one of the blue colonies, as before, and digested with EcoRi. The resulting two fragments substantially comigrated on agarose gel with the two fragments obtained from EcoRI-BamHI digestion of pPLa8, thereby confirming that the desired 47 base pair fragment from pAD3 had been correctly inserted at the reconstructed BamHI site in pPLa8. The plasmid was designated pPLa83 (Figure 4).

5. pPLa831 -- Bringing A BamHI Site Closer To The P_L Promoter In pPLa83

To bring a BamHI site closer to the P_L promoter in pPLa83, the EcoRI-EcoRI fragment was deleted by digestion of pPLa83 with EcoRI and religation at dilute DNA concentration (Figure 4). Transformation of the resulting recombinant DNA molecules into E. coli W6 (λ_{rex}) and growth on plates containing minimal medium supplemented

as before with X gal and kanamycin permitted selection of those clones no longer containing the lactose operator region. Restriction of the DNA from a selected transformant with BamHI-XhoI and a comparison of the migration of the resulting fragments with the two fragments obtained from EcoRI-XhoI digestion of pPLa8, confirmed that as expected the BamHI site in the modified plasmid was about 150 base pairs from the P_L promoter. The modified plasmid was designated pPLa831.

It should of course be understood that manipulations similar to those described in any of 3, 4 and 5 above could be employed to provide other endonuclease recognition sites less than 300 base pairs from the chosen promoter and operators in the vectors of this invention. Examples of such manipulations include those described below.

6. pPLa832 -- Insertion Of A HindIII Site Next To the BamHI Site of pPLa831

Plasmid pAD16 (a gift of H. Schaller) contains a 36 base pair fragment inserted in the BamHI site of pBR322 coding for the sequence: BamHI site-HindIII site-HindIII site-BamHI site. To insert this sequence at the BamHI site of pPLa831, pPLa831 and a 10-fold excess of pAD33 were cleaved with BamHI, religated and transformed into E. coli M5219 selecting for kanamycin resistance (Figure 4). Since there is no easy screening method to determine proper insertion of the desired BamHI fragment into pPLa831, analysis of the transformants that grew in the presence of kanamycin depended on restriction cleavage of individual, randomly chosen clones. Among 32 clones analysed, one was found that produced two fragments after cleavage with HindIII (Figure 4). The size of these fragments was indistinguishable on a 1.4% agarose gel from the fragments obtained after BamHI-HindIII cleavage or EcoRI-HindIII cleavage of pPLa831. This modified plasmid was designated pPLa832.

B. Vectors Containing The P_L Promoter In The Clockwise Orientation With Respect To The Origin Of Replication

1. pPLc2 -- Cloning of The P_L Carrying Fragment of pPLa832

An equimolar mixture of pBR322 and pPLa832 was cleaved with BamHI and subsequently with HindIII (Figure 4). The mixture was religated and transformed into M5219 selecting for resistance to carbenicillin. Since correctly prepared recombinant DNA molecules of this construction no longer include the intact gene for tetracycycline, the transformants were also screened for loss of resistance to tetracycline. The recombinant DNA molecule was isolated as before from selected transformants and analyzed by restriction. The selected plasmid contained a single HindIII site. Combined HindIII-BamHI digestion produced two fragments substantially comigrating on agarose gel with the two fragments produced by single EcoRI digestion. The presence of the P_L-carrying fragment was verified by HincII digestion. This enzyme cleaved the vector into three fragments, the sizes of which were consistent with the structure of the fragments shown in Figure 4. This plasmid was designated pPLc2, the "c" serving to indicate the clockwise orientation of the P_L promoter with respect to the origin of replication.

2. pPLc23 -- Removal of One EcoRI Site From pPLc2

Plasmid pPLc2 contains two EcoRI sites -- one derived from the parent pBR322 vector and one close to the BamHI site introduced by inserting the HindIII-BamHI fragments from pPLa832 (Figure 4). The EcoRI site derived from pBR322 was removed by cleaving pPLc2 with HindIII and XhoI followed by digestion with the Bal31 for 30 min at 25°C in 0.6 M NaCl, 12.5 mM each $CaCl_2$ and $MgSO_4$, 1 mM EDTA, 20 mM Tris-HCl (pH 8.1). Exonuclease Bal31 degrades 3'- and 5'-termini in a stepwise fashion (H. Gray et al. "Extracellular Nucleases of Pseudomonas Bal31. I. Characterization Of Single Strand-Specific Deoxyriboendonuclease

And Double-Strand Deoxyriboexonuclease Activities", Nucleic Acids Res., 2, pp. 1459-92 (1975)).

The mixture was extracted with phenol and chloroform, diluted to a DNA concentration of 1 µg/ml and ligated. Following ligation, the DNA was again cleaved with XhoI and HindIII to eliminate parental plasmid molecules and transformed into M5219 selecting for resistance to carbenicillin. One transformant was found which lacked a HindIII and a XhoI site. This plasmid contained a single EcoRI site and possessed three HincII sites (Figure 4). This latter property confirmed that the P_L region was still present. This plasmid was designated pPLc23.

To assess the extent of exonucleolytic degradation by the Bal31 enzyme, pPLc23 DNA was cleaved simultaneously with BamHI and PstI and the fragments sized on a 1.4% agarose gel. Compared to the PstI-BamHI fragment form the parent pPLc2 the PstI-BamHI fragment from pPLc23 displayed an over 800 base pairs deletion. Combined digestion with EcoRI-PstI-HaeII as compared to EcoRI-PstI cleavage confirmed that the HaeII site at the junction between the P_L-carrying fragment and the kanamycin fragment had been maintained.

3. pPLc236 -- Introduction of a HindIII Site In pPLc23

Plasmid pPLc23 contains unique EcoRI and BamHI sites located about 150 nucleotides downstream from the P_L promoter (Figure 4). A HindIII insertion site was introduced into pPLc23 by ligating the BamHI-HindIII-HindIII-BamHI fragment obtained from pPLa832 into the BamHI site of pPLc23. Transformants were obtained in M5219 and screened by restriction analysis for the presence of a HindIII site. The structure of a representative clone was confirmed by agarose gel electrophoresis of the fragments obtained after PstI-EcoRI, PstI-BamHI or PstI-HindIII digestion. The fragments obtained after each of

these combined digestions substantially comigrated on a 1.4% agarose gel showing that the EcoRI, BamHI and HindIII sites are localized in the immediate vicinity of each other. This plasmid was designated pPLc236 (Figure 4).

The larger part of plasmid pPLc236 is derived from pBR322 from the BamHI site at position 377 (J. Sutcliffe, supra) up to at least the start of the β-lactamase gene around position 4160 (J. Sutcliffe, supra). The remaining part is composed of 1) sequences derived from part of the kanamycin gene situated between the XhoI site and one HaeII end of this fragment; 2) a HaeII-BamHI fragment containing the P_L promoter, comprising about 300 nucleotides and derived from pPLa832; 3) a sequence coding for BamHI-HindIII-HindIII-BamHI sites.

4. pPLc28 -- Deletion From pPLc236

Overlapping the two adjacent HindIII sites in pPLc236 is a BalI site (not shown in Figure 4). The plasmid also contains a unique PvuII site at base pair 2067 of the pBR322 portion (J. Sutcliffe, supra) (Figure 4). The enzymes BalI and PvuII both produce flush ends. pPLc236 DNA was cleaved with BalI and PvuII and religated at low DNA concentration. Transformants were obtained in M5219 selecting for resistance to carbencillin. DNA of a representative clone was analyzed by restriction. BamHI cleavage produced a single fragment comigrating on 1.4% agarose gel with the larger part of pPLc236 after BamHI-PvuII cleavage. Combined digestion with either PstI-EcoRI, PstI-BamHI or PstI-HindIII in each case produced two fragments, the smaller of which substantially comigrated on a 1.4% agarose gel with a PstI-EcoRI fragment obtained from pPLc236. This plasmid was designated pPLc28 (Figure 4).

pPLc28, like the other plasmids described in accordance with this invention, may of course be further manipulated to insert other restriction sites. For example, a fragment containing the following: Xba restriction

site - Sal restriction site - Xba restriction site - Pst restriction site - Xba restriction site has been inserted in pPLc28 at the HindIII restriction site. This plasmid was designated pPLc2819. Another like manipulation afforded a plasmid containing the fragment Pst restriction site - Sal restriction - Xba restriction site - Sal restriction site - Xba restriction site inserted at the BamHI site of pPLc28. This plasmid was designated pPLc2833.

5. pPLc24 -- Insertion Of The Ribosome-Binding Site And The Amino-Terminal Part Of Bacteriophage MS2 Relicase Protein Into pPLc28

A 431 base pair EcoRI-BamHI fragment, coding for the ribosome binding site and the first 98 amino acid residues of the bacteriophage MS2 replicase gene was obtained from plasmid pMS2-7 (R. Devos et al., "Construction And Characterization Of A Plasmid Containing A Nearly Full-Size DNA Copy Of Bacteriophage MS2 RNA", J. Mol. Biol., 128, pp. 595-619 (1979)). This fragment was inserted into plasmid pPLc28 replacing the original EcoRI-BamHI fragment therein (Figure 5). The structure of the resulting plasmid, designated pPLc24, was verified by restriction analysis with EcoRI-BamHI and size comparison of the resulting fragments with those obtained after EcoRI-BamHI digestion of pMS2-7 and pPLc28. In pPLc24 translation of the MS2 replicase protein fragment runs colinearly with the transcription from the P_L promoter and hence is under P_L control.

BIOLOGICAL PROPERTIES OF HOST CELLS TRANSFORMED BY THE VECTORS OF THIS INVENTION

1. Stability At 28°C

Strains K12ΔHI or M5219 transformed with any of the above described vectors were grown at 28°C for 20 generations in LB medium without selection for the antibiotic resistance marker. Suitable dilutions of the

cultures were then plated at 28°C either in the presence or the absence of the desired antibiotic. In all cases the number of colonies obtained was the same regardless of the selection for the antibiotic resistance, demonstrating that the vectors were fully stable in these hosts at 28°C (see Table I, infra).

All vectors could also be transformed into a host strain lysogenic for bacteriophage λ. Such strains where the resident phage synthesizes a wild-type cI product were viable at elevated temperature (37°C). In contrast, non-lysogenic hosts could not be transformed with these vectors. Instead, the rare transformants obtained from these experiments invariably contained vectors with deletions removing all or most of the P_L region.

2. Behavior Of Cells Containing P_L Vectors After Prolonged Induction At 42°C

The efficiency of plating at 42°C of strains K12ΔHI and M5219 transformed with the vectors of this invention was determined either in the presence or absence of antibiotic selection.

Vectors having the P_L inserted in the clockwise orientation with respect to the origin of replication (pPLc-type) behaved similarly. The results obtained with pPLc236 are listed in Table I, infra. Strain K12ΔHI transformed with pPLc236 plated equally well at 42°C as at 28°C regardless of whether antibiotic selection was applied or not. Strain M5219 transformed with pPLc236 plated on non-selective plates with an efficiency of 1. However, when antibiotic selection was applied, the efficiency of plating dropped at least 1000-fold. Colonies obtained at 42°C on non-selective plates no longer carried resistance to the antibiotic marker.

Vectors having the P_L inserted in the anticlockwise orientation with respect to the origin of replication (pPLa-type) displayed a more complex pattern of colony

formation at 42°C. Transformants of strain M5219 did not form colonies at 42°C even in the absence of antibiotic selection (the efficiency of plating was less than 10^{-3}; Table I). The behavior of transformants of strain K12ΔHI at 42°C depended on the nature of the vector present. For example, whereas transformants containing pPLa832 invariably displayed at least a 1000-fold reduction of plating efficiency, both with and without antibiotic selection, transformants containing pPLa23 or pPLa2311 displayed plating efficiencies ranging from 1 to 10^{-3}, frequently with wide heterogeneity in colony size.

Therefore, expression of pPLa-type vectors at 42°C causes interference with host metabolism, making the cells unable to survive at this high temperature, even in the absence of selection for the plasmid. This effect is most pronounced using M5219 hosts. Conversely, pPLc-type vectors do not interfere directly with host cell metabolism because 100% survivial of induced cells in the absence of selective pressure is observed. Continued transcription from the P_L promoter concomitant with expression of the N gene in M5219 may, however, result in inhibition of vector replication in M5219 strains. This is illustrated by the inability of such cells to grow at 42°C on selective plates.

TABLE I

Strain	Vector	Plating efficiency* 28°C Without selection	28°C With selection	42°C Without selection	42°C With selection
K12ΔHI	none	1	-	1	-
	pPLa23	1	1	1 to $\leq 10^{-3}$	1 to $\leq 10^{-3}$
	pPLa2311	1	1	1 to $\leq 10^{-3}$	1 to $\leq 10^{-3}$
	pPLa832	1	1	$\leq 10^{-3}$	$\leq 10^{-3}$
	pPLc236	1	1	1	1
M5219	none	1	-	1	-
	pPLa23	1	1	$\leq 10^{-3}$	$\leq 10^{-3}$
	pPLa2311	1	1	$\leq 10^{-3}$	$\leq 10^{-3}$
	pPLa832	1	1	$\leq 10^{-3}$	$\leq 10^{-3}$
	pPLc236	1	1	1	$\leq 10^{-3}$

* Bacterial cultures were grown to saturation in LB medium at 28°C in the presence of antibiotic. Suitable dilutions were plated either in the presence or absence of antibiotic and incubated at 28°C or 42°C. The number of colonies obtained was determined.

EXPRESSION OF GENES IN THE VECTORS OF THIS INVENTION

1. General Procedure

The vectors of this invention may be usefully employed to produce a variety of polypeptides and proteins by inserting DNA sequences comprising genes coding for the desired polypeptides or proteins into the vectors at one of the endonuclease recognition sites adjacent to the promoter and operator, transforming appropriate hosts with vectors containing those inserted DNA sequences, culturing the hosts and collecting the polypeptides or protein products. Examples of such polypeptides and proteins include leukocyte interferon, insulin, antigens of hepatitis, antigens of foot and mouth disease, fibroblast interferon, human growth hormone, immune interferon and a variety of other prokaryotic, eukaryotic and viral enzymes, hormones, polypeptides, antigens and proteins.

To illustrate these processes, the synthesis of specific gene products in the vectors of this invention was monitored by pulse-labelling of induced cells and analysis of the labelled proteins by polyacrylamide gel electrophoresis.

Cells transformed with the vectors of this invention were grown in LB medium without antibiotic at 28°C to a density of 2 x 10^8/ml. The cells were collected by centrifugation and resuspended in the original volume of a medium consisting of 19 mM NH_4Cl, 86 mM NaCl, 42 mM Na_2HPO_4, 1 mM $MgSO_4$, 0.2% glucose, 0.05% casamino acids (Difco), 0.01% yeast extract and 50 µg/ml/ L-tryptophan for labelling of the cells with ^{14}C-amino acid mixture or the above medium except for a substitution of 5% methionine assay medium (Difco) for the casamino acids and yeast extract for labelling of the cells with ^{35}S-methionine. Incubation at 28°C was continued for 60 min. One-half of the culture was then shifted to 42°C. At various times after induction, aliquots from the 28°C and 42°C cultures

were labeled with ^{14}C-amino acid mixture or with ^{35}S-methionine (Amersham).

Incorporation of label was terminated by phenol extraction. The synthesized proteins were precipitated from the phenol layer by addition of 5 vol ethanol and redissolved in 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8). Samples were boiled for 5 min, centrifuged at 12000 x g and electrophoresed in SDS-containing polyacrylamide gels (10% to 15% acrylamide) according to the procedure of U.Laemmli, "Cleavage Of Structural Proteins During the Assembly Of The Head Of Bacteriophage T4", Nature, 227, pp. 680-82 (1970). Following electrophoresis, the gels were prepared for fluorography according to the method of W. Bonner & R. Laskey, "A Film Detection Method For Tritium-Labelled Proteins And Nucleic Acids In Polyacrylamide Gels", Eur. J. Biochem., 46, pp. 83-88 (1974) except that EN^{3}HANCE (NEN) was employed instead of PPO-DMSO.

2. Prokaryotic Genes

(a) The β-Lactamase Gene

pPLa23 (Figure 3) includes the β-lactamase gene in the sense orientation downstream from the P_L promoter. Therefore, production of the protein coded for by the β-lactamase gene can be monitored as an indication of the efficiency of the vector in expressing prokaryotic genes.

Transformants of K12ΔHI and M5219 with pPLa23 -- E. coli K12ΔHI (pPLa23) and E. coli M5219 (pPLa23) -- were prepared as described previously and their protein synthesis monitored. The results are displayed in Figure 8. There, it can be seen that a dramatic increase in the rate of synthetis of two proteins with apparent molecular weights of 27.5K and 30K, respectively, occurred shortly after induction of the transformants at 42°C. The sizes of these expressed proteins are consistent with the expected length of mature β-lactamase and its precursor (J. Sutcliffe, supra). Moreover, induced synthesis of

these proteins was paralleled by an increasing enzymatic activity of β-lactamase as determined by the method of O'Callaghan et al., "Novel Method For Detection Of β-Lactamases By Using A Chromogenic Cephalosporin Substrate", Antimicrobial Agents and Chemotherapy, 1, pp. 283-88 (1972) and both proteins were specifically precipitated by anti-β-lactamase serum. As a control, the protein synthesis in hosts not transformed with vector pPLa23 was monitored. Synthesis of neither of the two above described proteins was observed from these non transformed hosts.

As shown in Figure 8, the overall pattern of protein synthesis in these transformants is very similar at 28°C and 42°C. However, the rate of synthesis of some proteins appears to be altered significantly by shifting the cells to 42°C. Similar behavior has been observed in cells not transformed with pPLa23. In addition, as shown in Figure 8, the relative amount of the larger of the two β-lactamase related proteins -- the unprocessed precursor for β-lactamase -- becomes greater with time after induction. This skewing towards a build-up of precursor protein may indicate a saturation of the β-lactamase processing machinery of the cell.

To determine the percentage synthesis of β-lactamase as compared to total de novo protein synthesis of the transformant, the protein bands for the β-lactamase (27.5K) and its precursor (30K) were excised from the dried gel and their radioactivity compared to the total radioactivity applied to the gel. These results are displayed in Table II.

TABLE II

Percentage Synthesis Of
β-lactamase As Compared To
Total De Novo Protein Synthesis

Minutes after induction at 42°C	Strain	
	K12ΔHI	M5219
0-10	8%	9%
10-20	9%	16%
20-30	10%	25%
30-40	16%	24%
40-50	23%	30%
50-60	27%	-
60-70	30%	-
70-80	33%	-
control at 28°C	5%	4%

As shown in Table II, synthesis of β-lactamase and its precursor reaches a maximum level of about 30% of total de novo protein synthesis in both host cell strains. However, the kinetics of reaching this level are different for the two strains -- strain K12ΔHI lags about 20 min behind strain M5219 in attaining the 30% level. While not wishing to be bound by theory, it may be that the N gene product, co-produced upon induction of strain M5219 but absent in strain K12ΔHI, may overcome certain transcription slow-down signals in the DNA sequences downstream from the P_L promoter and thereby speed β-lactamase synthesis in strain M5219.

To determine the rate of total protein synthesis in these transformants, total radioactivity incorporated

during a specific time interval was determined and compared to that incorporated during the 0-10 min interval (i.e., that initial interval being arbitrarily chosen as 100% for a reference). The results are displayed in Table III.

TABLE III

Rate Of Total Protein Synthesis

Minutes after induction at 42°C	Strain	
	K12ΔHI	M5219
1-10	100%	100%
10-20	104%	92%
20-30	134%	56%
30-40	113%	31%
40-50	120%	10%
50-60	113%	7%
60-70	96%	3%
70-80	96%	3%
150-160	20%	-

As shown in Table III, total protein synthesis in E. coli M5219 (pPLa23) is rapidly shut down after induction. This is consistent with the previously observed failure of M5219 transformants to survive 42°C. No similar inhibition of protein synthesis is observed in E. coli M5219 (pBR322). A substantial reduction in total protein synthesis is also observed in E. coli K12ΔHI (pPLa23) after prolonged incubation at 42°C. However, these cells are able to survive 42°C temperatures.

(b) The Tryptophan Synthetase A Gene

(i) pPLa23

An EcoRI fragment (5300 b.p.) containing the trp A cistron of Salmonella typhimurium was obtained from pES9 (E. Selker et al., "Mitomycin C Induced Expression Of trp A Of Salmonella tryphimurium Inserted Into The Plasmid ColE1", J. Bacteriology, 129, pp. 388-94 (1977)) and inserted into pPLa23 at its EcoRI site. Two representative plasmids having this fragment inserted in either of the two possible orientations with respect to the direction of the P_L promoter were designated pPLa23trpA$_1$ and pPLa23trpA$_2$.

Induction profiles of strain K12ΔHI containing either pPLa23trpA$_1$ or pPLa23trpA$_2$ are shown in Figure 9. A major protein of about 25000 daltons was induced by pPLa23trpA$_1$ but was absent from induced cells containing pPLa23trpA$_2$. The observed molecular weight of this protein is consistent with the theoretical value (28500) predicted from the nucleotide sequence of the S. typhimurium trp A gene (B. Nichols & C. Yanofsky, "Nucleotide Sequences Of trp A Of Salmonella tryphimurium And Escherichia coli: An Evolutionary Comparison", Proc Natl. Acad. Sci. U.S.A., 76, pp. 5244-48 (1979). Moreover, enzyme activity consistent with the presence of a trp A gene product, as determined according to the procedures of O. Smith & C. Yanofsky "Enzymes Involved In The Biosynthesis Of Tryptophan", Methods in Enzymology, 5, pp. 794-806, (1962) increased in parallel with the accumulation of this induced protein.

After prolonged induction of both pPLa23trpA$_1$ and pPLa23trpA$_2$ a protein with an approximate molecular weight of 18K is synthesized (Figure 9). The percentage synthesis of this protein as compared to total de novo protein synthesis of the transformant is independent of the orientation of the EcoRI trp A fragment with respect to the direction of transcription from the P_L promoter.

Therefore, presumably the synthesis of this protein is controlled by a, perhaps slightly temperature-dependent, bacterial promoter present on the 5300 base pair EcoRI trp A fragment whose coding capacity is indeed much larger than needed for trp A. (E. Selker, supra).

The percentage synthesis of trp A as compared to the total de novo protein synthesis in the transformant was determined substantially as described previously for β-lactamase. The results are displayed in Table IV. Again, trp A synthesis reached a maximum level of about 30% of total de novo synthesis.

TABLE IV

Percentage Synthesis Of trp A As Compared To Total De Novo Protein Synthesis

Minutes after induction at 42°C	K12ΔHI /pPLa23A$_1$	K12ΔHI /pPLa23A$_2$
0-10	3%	2%
30-50	4%	2%
60-80	14%	1%
90-110	21%	2%
120-140	24%	2%
150-170	33%	2%
control at 28°C	2%	3%

(ii) pPLa2311

A recombinant DNA molecule identical to pPLa23trpA$_1$, but based on pPLa2311, was also prepared

substantially as described previously. K12ΔHI transformants with pPLa2311trpA$_1$, behaved similarly to those of pPLa23trpA$_1$ (except that in this experiment the maximum level of total de novo synthesis was only 20% after 150 min). Again, prolonged induction led to a net decrease in total protein synthesis.

(iii) pPLc23

The EcoRI fragment of pES9 has one end situated within the trp B gene and contains a single SalI site about 2500 base pairs from the EcoRI site (E. Selker et al., supra). Since the trp A gene does not contain a SalI site (B. Nichols & C. Yanofsky, supra), the trp A gene must be located completely in that portion of the EcoRI fragment of pES9 extending from the first EcoRI site to the SalI site. Therefore, the previously-prepared EcoRI fragment of pES9 was digested with SalI and the resulting fragment inserted into pPLc23 as a replacement to the EcoRI-SalI fragment therein (Figure 4). Based on the observed direction of translation of the trp A gene in pPLa23trpA$_1$, and pPLa23trpA$_2$, the pPLc23-based recombinant DNA molecule having the trp A gene colinear with transcription from the P_L promoter was designated pPLc23trpA$_1$.

Upon induction (42°C) of E. coli K12ΔHI (pPLc23trpA$_1$), trp A was synthesized to a maximum level of about 40% of total de novo protein synthesis after 3 h of induction. Moreover, this high level of de novo synthesis was maintained for 2 h (Figure 10). These results are displayed in Table V, infra. Therefore, in contrast to the behavior of the pPLa-type vectors, the protein synthesis of the pPLc-type transformants does not decrease until up to 5 h after induction.

TABLE V

Minutes after induction at 42°C	Percentage synthesis of trp A*	Rate of total protein synthesis
30-50	11%	100% (reference)
60-80	17%	198%
120-140	31%	228%
180-200	41%	162%
240-260	36%	205%
300-320	39%	213%
control at 28°C (300-320)	3%	-

* As compared to total de novo protein synthesis

The actual amount of induced protein accumulating in the above transformants was also measured by continuous labelling of the induced cells. E. coli K12ΔHI (pPLc23trpA$_1$) was grown at 28°C in LB medium to a density of 1X10^7 cells/ml. The cells were then labelled with 10 µCi ^{14}C-amino acid mixture. At a culture density of 4 x 10^7 cells/ml, the cells were shifted to 42°C and incubation continued. When the culture reached saturation (6 h after induction), the proteins were extracted from the cells and separated on SDS-polyacrylamide gels. The percentage of radioactivity incorporated in the trp A band was determined. Under the conditions used it can be assumed that the cells have been uniformly labelled so that the radioactivity incorporated in a protein reflects the actual amount of that protein present in the cell. The trp A protein was found to account for 10% of total cell proteins.

This 10% concentration of trp A in the total cell proteins of E. coli K12ΔHI (pPLc23trpA$_1$) also serves to demonstrate the important differences between the λ P_L containing vectors of H. Bernard et al., supra, and those of the present invention. In contrast to the 10% actual trp A concentration afforded by vectors of this invention, H. Bernard et al. report only a 6.6% concentration of trp A -- estimated on the basis of trp A enzymatic activity and an assumed specific activity for the protein. It is also to be noted that the 6.6% trp A concentration reported by H. Bernard et al. was observed with vectors which also included an active N-gene and therefore presumably transcription was had in the presence of the anti-terminating N gene product. Only a 2% trp A concentration was reported by H. Bernard et al. with a vector that did not include an active N-gene. In contrast, the 10% trp A concentration observed with improved vectors of this invention was had in the absence of N-gene products. Therefore, the present vectors and methods constitute a marked improvement over those vectors and methods described in the art.

(c) The Bacteriophage MS2 Replicase Protein Gene

Plasmid pMS2-7 contains a nearly full-size copy of the genome of the RNA bacteriophage MS2 (R. Devos et al., supra). The phage replicase gene (R) is contained within an EcoRI-PstI fragment. This fragment was inserted into pPLa2311 by simple replacement of the EcoRI-PstI fragment of this vector. Transformants E. coli K12ΔHI (pPLa2311R$_1$) were screened for sensitivity to carbenicillin since the gene for ampicillin resistance is no longer intact in pPLa2311R$_1$. The identity of the inserted fragment was established by coelectrophoresis on agarose gels with the known fragments from pMS2-7 DNA. In pPLa2311R$_1$, transcription of the MS2 replicase protein runs colinearly with transcription from the P_L promoter.

Induced cells of E. coli K12ΔHI (pPLa2311R$_1$) synthesize a protein with an apparent molecular weight of

59K (Figure 11). The size of this protein is consistent with the 60692 daltons molecular weight, calculated for the MS2 replicase from sequence data of the viral RNA (W. Fiers et al., "Complete Nucleotide Sequence Of Bacteriophage MS2 RNA: Primary And Secondary Structure Of The Replicase Gene", Nature, 260, pp. 500-507 (1976)).

The presence of functional MS2 replicase protein in the protein products of cells transformed with pPLa2311R$_1$ was also verified by complementation analysis with MS2 amber mutants. This analysis confirmed that cells transformed with pPLa2311R$_1$ produced a product that specifically complemented the product of an MS2 mutant carrying a lesion in the replicase gene and that cells not transformed with pPLa2311R$_1$ did not complement the product of such mutant.

With respect to MS2 replicase protein synthesis, both E. coli K12ΔHI (pPLa2311R$_1$) and E. coli M5219 (pPLa2311R$_1$) behaved similarly -- after 30 min induction the percentage synthesis of MS2 replicase was 29% of total de novo protein synthesis, with the level of protein synthesis dropping rapidly upon further induction (Figure 11). Since such decrease in the level of synthesis was not observed in the synthesis of β-lactamase or trp A, the reduction may be caused by a peculiar property of the MS2 replicase. For example, the observed tendency of phage replicase to bind to its own mRNA at a site near the middle of the cistron (Meyer et al., "The Binding Sites Of Qβ RNA", Experienta, 31, pp. 143 et seq. (1975)) may interfere with further translation of the complexed mRNA.

3. Eukaryotic Genes

(a) The Small-t Antigen Of Simian Virus 40 Gene

A HindIII DNA fragment containing the complete coding sequence for the SV40 small-t antigen (G. Volckaert et al., "Nucleotide Sequence Of The Simian Virus 40 Small-t Gene", Proc. Natl. Acad. Sci. U.S.A., 75,

pp. 2160-64 (1978)) was inserted into the HindIII site of pBR322 (Figure 12). The orientation of the insert was determined by restriction analysis based on the presence of an asymmetrically-located TaqI site. From this hybrid DNA molecule an EcoRI-BamHI fragment encompassing the above-mentioned HindIII fragment and portions of pBR322 was excised and inserted into pPLc28, as a replacement for its EcoRI-BamHI fragment, such that the sense of translation of the small-t antigen runs colinearly with transcription from the P_L promoter (Figure 12). The resulting recombinant DNA molecule was designated pPLc28SV$_t$5.

To shorten the distance between the P_L promoter and the initiating codon (ATG) of the gene coding for small-t antigen, pPLc28SV$_t$5 was modified to eliminate the EcoRI-HindIII fragment between the gene and the P_L promoter. These manipulations are depicted in Figures 12 and 13. They consisted of cleaving pPLc28SV$_t$5 with ClaI, chewing back the 3' end of the DNA in two separate steps using the 3' exonuclease activity of T4 DNA in the presence of GTP and TTP, respectively, further treatment with S1 nuclease, adding EcoRI linkers to the blunt end, cleaving the fragment with EcoRI and religating the complementary ends.

Transformation of E. coli K12ΔH1 with these modified hybrid DNA molecules and induction afforded expression of fairly large amounts of a protein with an apparent molecular weight of 14K (Figure 14). This protein was not produced without induction and was not produced by host cells that had not been transformed with vectors containing SV40 DNA.

Although authentic small-t antigen has a molecular weight of 19K and the protein produced in these transformed cells was precipitated only very poorly by antibodies raised against the large-T antigen of SV40, two dimensional finger prints (electrophoesis at pH 3.5, followed by chromatography in butanol/acetic acid/pyridine/water (15:3:10:12)) of tryptic peptides derived from this

protein and authentic small-t antigen confirmed that the two were related. While not wishing to be bound by theory, it may be that the secondary structure of the mRNA starting at the P_L promoter is such that initiation at an internal initiating codon of small-t antigen is favored over initiation at the true start signal. This hypothesis is also consistent with the secondary structure that could be derived, using the procedures of D. Iserentant & W. Fiers, "Secondary Structure Of mRNA And Efficiency Of Translation Initiation", Gene, 9, pp. 1-12 (1980), from the nucleotide sequences of some of these SV_t containing vectors.

One of the modified pPLc28SV_t5 recombinant DNA molecules, prepared as above, expressed minor amounts of a 17K component in addition to the major 14K protein component. This molecule was designated pPLcSV_t5-37. While the presence of the 17K protein could only be initially detected by specific immunoprecipitation with large-T antiserum, further modification of the molecule permitted enhanced synthesis of the 17K component. This modification, which consisted of cleaving pPLc28SV_t5-37 with EcoRI, extending the recessed 3' ends with DNA polymerase I (K. Backman et al., supra) and religating the blunt ends, may have changed the secondary structure of the mRNA. Hosts transformed with pPLc28SV_t5-37-9 afforded approximately 4% of their total de novo protein synthesis as a 17K protein component upon induction. These results are displayed in Figure 14. As shown in line c of Figure 14, the 17K component was immunoprecipitated with serum from an SV40-tumor-bearing hamster to substantially the same extent as authentic small-t antigen grown in SV40-infected African green monkey kidney cells.

(b) The Human Fibroblast Interferon (HFIF) Gene

As described in British patent application 80.18701 filed June 6, 1980, the gene coding for human fibroblast interferon was inserted into vectors pPLa8 and

pPLc24 to produce recombinant DNA molecules that are capable in transformed hosts after appropriate induction of expressing proteins having an antiviral, physio-chemical, immunlogical and biological activity closely corresponding to authentic human fibroblast interferon.

(c) An FMDV Antigen Gene

As described in British patent application 80.26661, filed August 15, 1980, a DNA sequence coding for a polypeptide displaying the specificity of FMD viral antigens was inserted into vector pPLc24 to produce recombinant DNA molecules that are capable in transformed hosts after appropriate induction of expressing polypeptides having the specificity of FMD viral antigens.

Microorganisms and vectors prepared by the processes described herein are exemplified by cultures deposited in the American Type Culture Collection, Rockville, Maryland, United States on September 8, 1980 and identified as PL-A to PL-D:

A. E. coli M5219 (pPLa2311)
B. E. coli K12ΔHI (pPLa8)
C. E. coli K12ΔHI (pPLc28)
D. E. coli M5219 (pPLc24)

These cultures were assigned accession numbers ATTC 31694-31697, respectively.

In addition, microorganisms and vectors prepared by the processes described herein and also containing inserted DNA sequences for expression therein are exemplified by cultures deposited in culture collection Deutsche Sammlung von Mikroorganismen in Gottingen, West Germany and identified as follows:

HFIF-D: E. coli M5219 (G-pPLa-HFIF-67-12) [DSM 1851]
HFIF-E: E. coli K12ΔHI (G-pPLa-HFIF-67-12) [DSM 1852]
HFIF-F: E. coli M5219 (G-pPLa-HFIF-67-12Δ19) [DSM 1853]
HFIF-G: E. coli M5219 (G-pPLc-HFIF-67-8) [DSM 1854]
FMDV-A: E. coli W6 (λ_{rex} - pPL-VP1-1) [DSM 1879]
FMDV-B: E. coli NF1 ($\lambda N^- cro^- cI_{ts}$-pPL-VP1-1) [DSM 1880]
FMDV-C: E. coli NF1 ($\lambda N^- cro^- cI_{ts}$-pPL-VP1-5) [DSM 1881].

Cultures HFIF-D - HFIF-G were deposited on June 5, 1980.
Cultures FMDV-A - FMDV-C were deposited on July 31, 1980.

While we have hereinbefore presented a number of embodiments of this invention, it is apparent that our basic construction can be altered to provide other embodiments which utilize the processes and compositions of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than the specific embodiments which have been presented hereinbefore by way of example.

CLAIMS

1. A vector comprising at least one DNA sequence comprising at least one promoter and operator derived from bacteriophage λ, characterized by at least one endonuclease recognition site located less than about 300 base pairs from that portion of said DNA sequence comprising said promoter and operator.

2. The vector of claim 1, characterized by the absence of active cro gene or active N gene.

3. The vector of claim 1 or 2, characterized in that said promoter and operator is P_LO_L or P_RO_R.

4. The vector of any one of claims 1 to 3, characterized in that said recognition site comprises EcoRI, BamHI, HindIII, PstI, Xba or Sal.

5. The vector of any one of claims 1 to 4, characterized in that said recognition site is located less than about 150 base pairs from that portion of said DNA sequence comprising said promoter and operator.

6. The vector of any one of claims 1 to 5, characterized by including a ribosome binding site.

7. The vector of claim 6, characterized in that said ribosome binding site is derived from bacteriophage MS2 replicase.

8. The vector of any one of claims 1 to 7, characterized by including in one of said endonuclease recognition sites a DNA sequence coding for a eukaryotic, prokaryotic or viral protein, polypeptide, enzyme, hormone, antigen or fragment thereof.

9. A method for producing an improved cloning vector, characterized by inserting into an existing cloning vehicle at least one DNA sequence comprising at least one promoter and operator derived from bacteriophage λ and

consisting of P_RO_R or P_LO_L and providing in said cloning vehicle at least one endonuclease recognition site less than about 300 base pairs from that portion of said DNA sequence consisting of said promoter and operator.

10. The method of claim 9, characterized by inactivating any active cro gene or any active N gene located in said DNA sequence.

11. The method of claim 9 or 10, characterized in that said recognition site comprises EcoRI, BamHI, HindIII, PstI, Xba or Sal.

12. The method of any one of claims 9 to 11, characterized in that said recognition site is located less than about 150 base pairs from that portion of said DNA sequence consisting of said promoter and operator.

13. The method of any one of claims 9 to 12, characterized by inserting a ribosome binding site in said vector.

14. The method of claim 13, characterized in that said ribosome binding site is derived from bacteriophage MS2 replicase.

15. A method for producing a recombinant DNA molecule, characterized by inserting a DNA sequence coding for a eukaryotic, prokaryotic or viral protein, polypeptide, enzyme, hormone, antigen or fragment thereof into at least one endonuclease recognition site of a vector comprising (1) at least one DNA sequence comprising at least one promoter and operator derived from bacteriophage λ and consisting of P_RO_R or P_LO_L and (2) said at least one endonuclease recognition site which is located less than about 300 base pairs from that portion of said DNA sequence comprising said promoter and operator.

16. A method for producing a polypeptide, characterized by culturing a host transformed with a vector according

to claim 8, and collecting said polypeptide.

17. The method of claim 16, characterized in that said polypeptide comprises leukocyte interferon, fibroblast interferon, immune interferon, insulin, human growth hormone, animal growth hormone, antigens of hepatitis, foot and mouth disease and other viruses, and other prokaryotic, eukaryotic and viral enzymes, hormones, polypeptides, proteins or amino acids.

FIG. 1

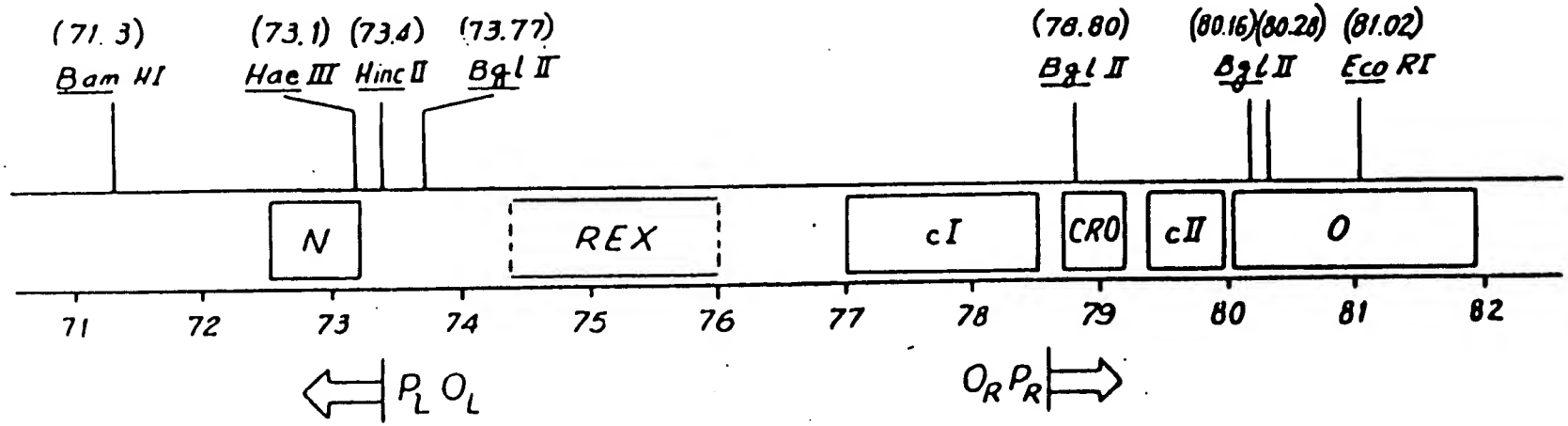

(71.3)
Bam HI
(73.1)
Hae III
(73.4)
Hinc II
(73.77)
Bgl II
(78.80)
Bgl II
(80.16)(80.28)
Bgl II
(81.02)
Eco RI
N
REX
cI
CRO
cII
O
71
72
73
74
75
76
77
78
79
80
81
82
P_L O_L
O_R P_R

FIG. 2

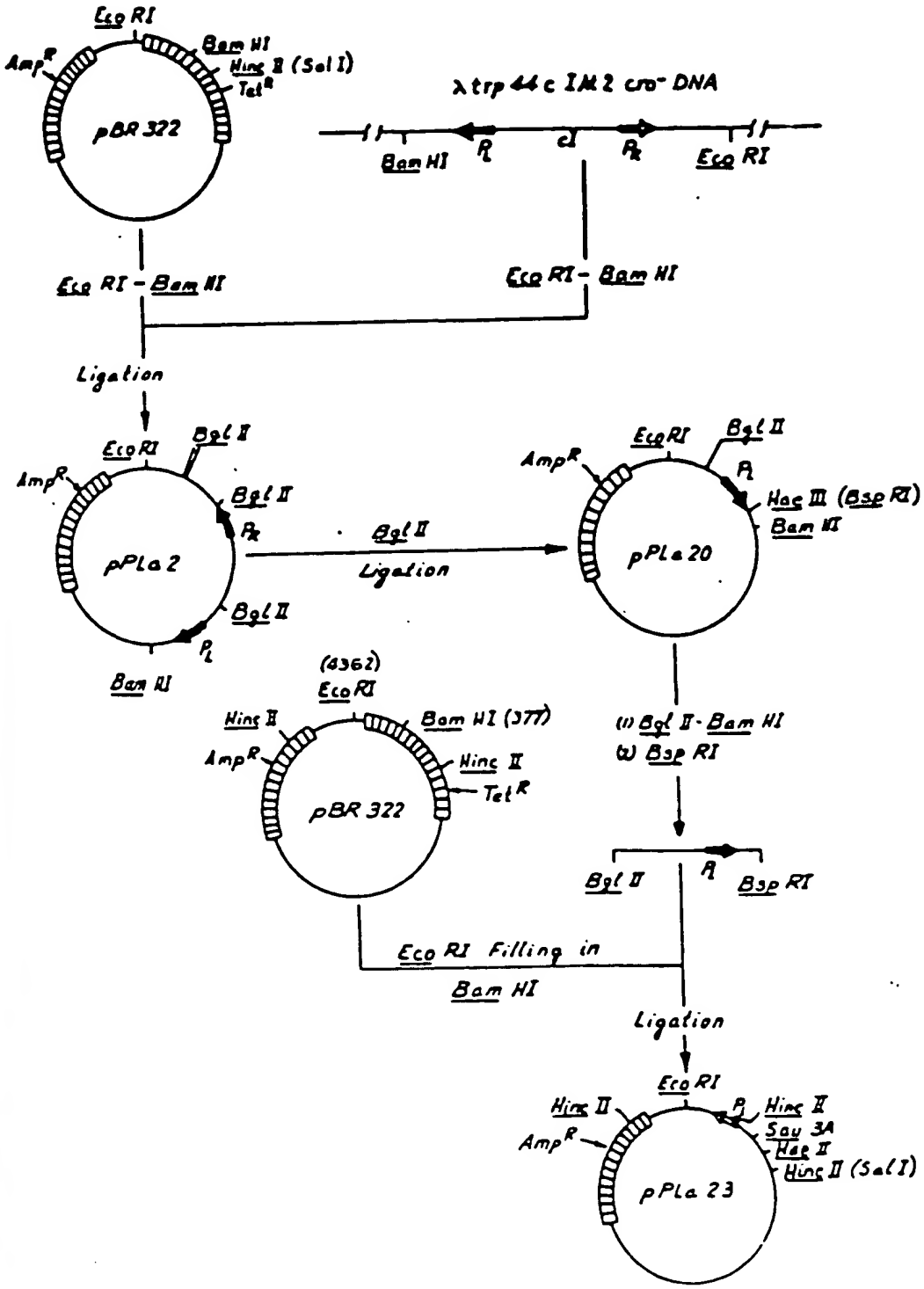
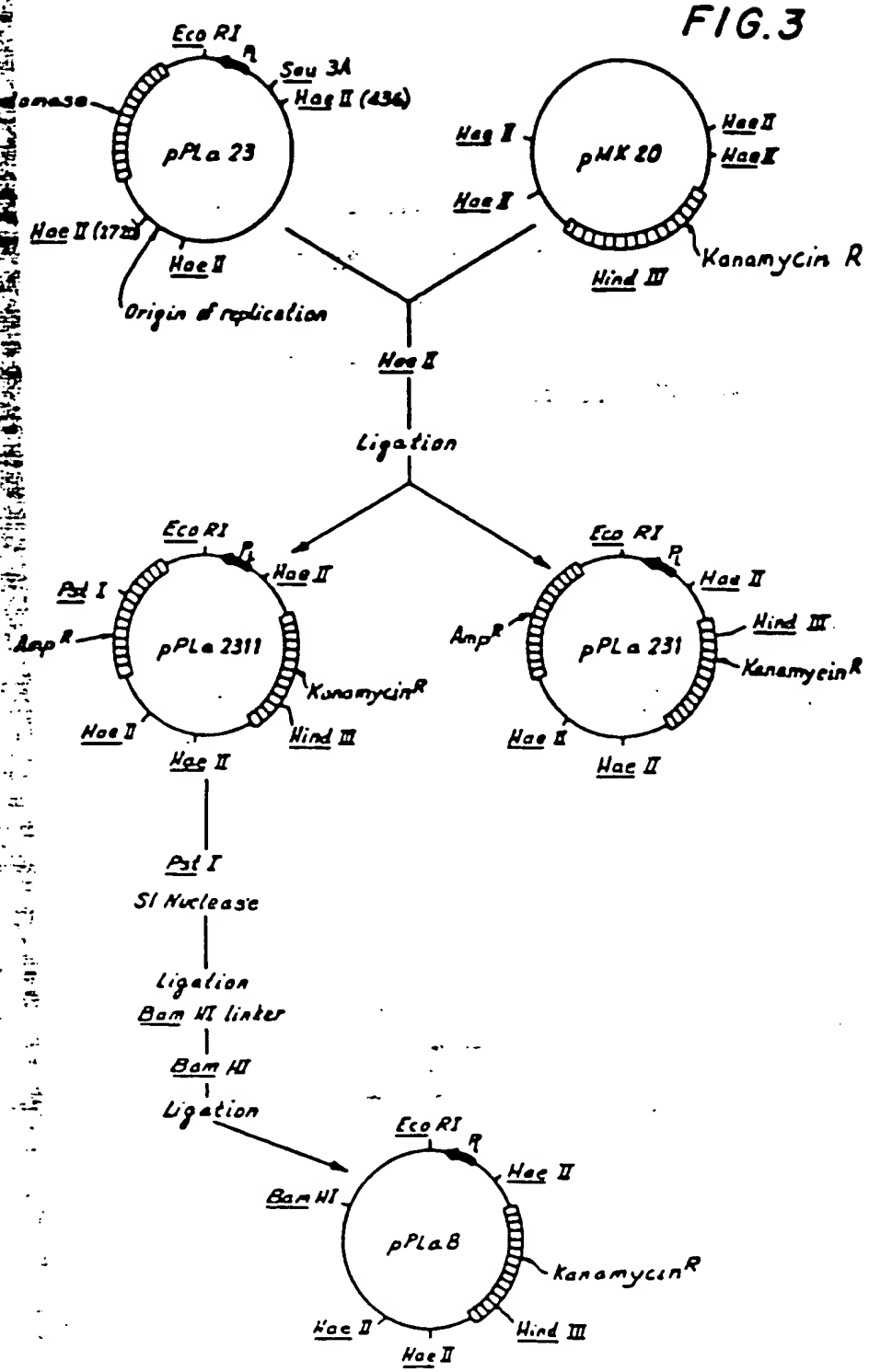

Eco RI
Bam HI
Hind III (Sal I)
Amp^R
Tet^R
pBR 322
λ trp 44 c IM 2 cro⁻ DNA
Bam HI
P_L
cI
P_R
Eco RI
Eco RI - Bam HI
Eco RI - Bam HI
Ligation
Eco RI
Bgl II
Amp^R
Bgl II
P_R
pPLa 2
Bgl II
P_L
Bam HI
Bgl II
Ligation
Eco RI
Bgl II
Amp^R
P_L
Hae III (Bsp RI)
Bam HI
pPLa 20
(4362)
Eco RI
Hind III
Bam HI (377)
Amp^R
Hind III
Tet^R
pBR 322
(i) Bgl II - Bam HI
(ii) Bsp RI
Bgl II
P_L
Bsp RI
Eco RI Filling in
Bam HI
Ligation
Eco RI
Hind III
P_L
Hind III
Sau 3A
Hae II
Hind III (Sal I)
Amp^R
pPLa 23

FIG.3


Eco RI R
Sau 3A
Hae II (436)
pPLa 23
Hae II (172)
Hae II
Origin of replication
Hae II
Hae II
pMK 20
Hae II
Hae II
Kanamycin R
Hind III
Hae II
Ligation
Eco RI R
Hae II
Pst I
Amp R
pPLa 2311
Kanamycin R
Hae II
Hind III
Hae II
Eco RI R
Hae II
Hind III
Amp R
pPLa 231
Kanamycin R
Hae II
Hae II
Pst I
S1 Nuclease
Ligation
Bam HI linker
Bam HI
Ligation
Eco RI R
Hae II
Bam HI
pPLa8
Kanamycin R
Hae II
Hind III
Hae II

4/14

FIG. 4

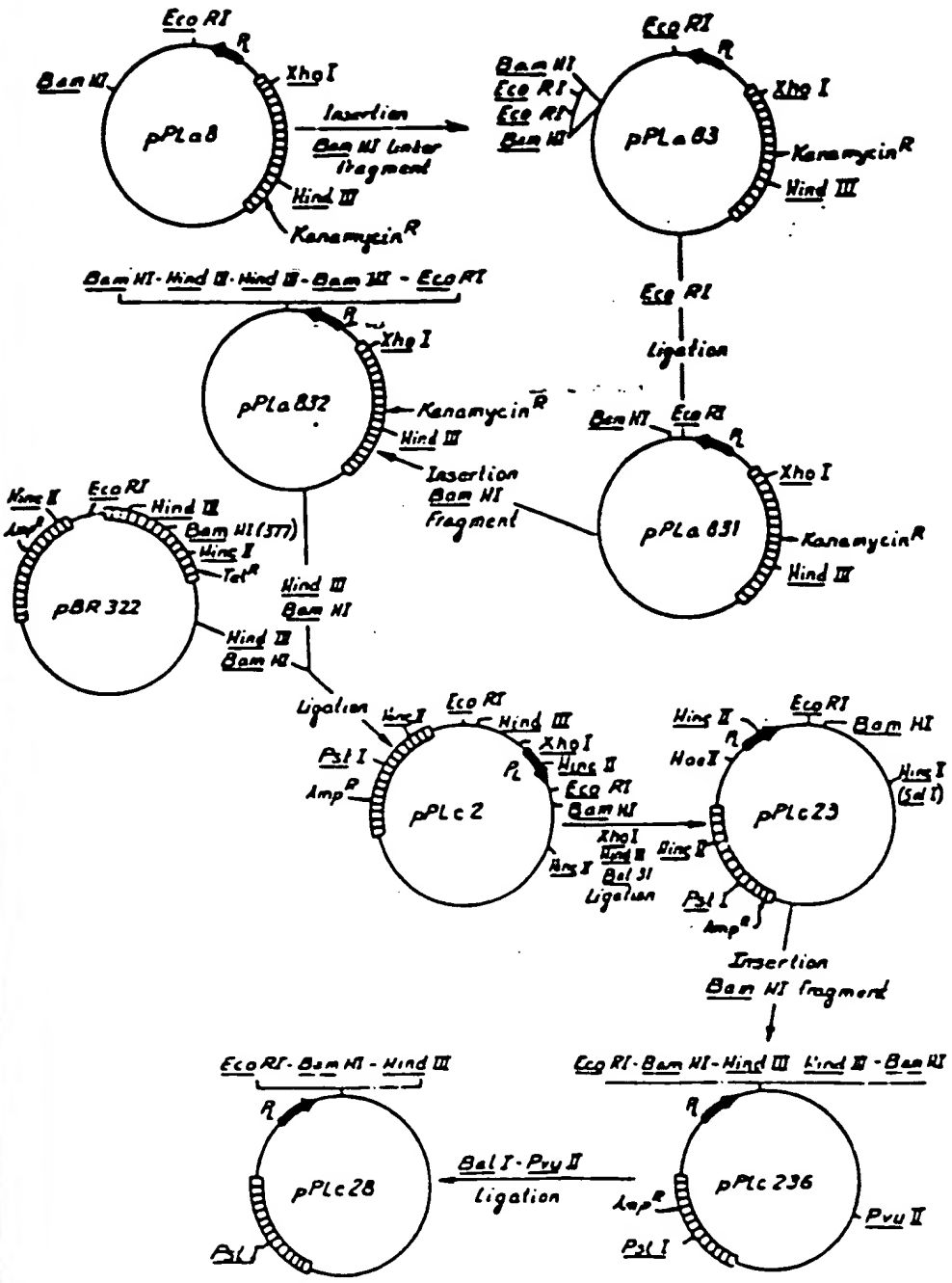

Eco RI
R
Xho I
Bam HI
pPLa8
Insertion
Bam HI Linker fragment
Hind III
Kanamycin R
Bam HI
Eco RI
Eco RI
Bam HI
pPLa83
Xho I
Kanamycin R
Hind III
Bam HI - Hind III - Hind III - Bam HI - Eco RI
Eco RI
Ligation
pPLa832
Xho I
Kanamycin R
Hind III
Insertion
Bam HI
Fragment
Bam HI
Eco RI
R
Xho I
pPLa831
Kanamycin R
Hind III
Hinc II
Eco RI
Hind III
Amp R
Bam HI (577)
Hinc II
Tet R
pBR322
Hind III
Bam HI
Hind III
Bam HI
Ligation
Hinc II
Eco RI
Hind III
Xho I
Pst I
Hinc II
Amp R
pPLc2
Eco RI
Bam HI
Hinc II
Hae II
R
Hinc II (Sal I)
pPLc23
Xho I
Hind III
Bal 31
Ligation
Hinc II
Pst I
Amp R
Insertion
Bam HI fragment
Eco RI - Bam HI - Hind III
Eco RI - Bam HI - Hind III Hind III - Bam HI
R
pPLc28
Bal I - Pvu II
Ligation
pPLc236
Amp R
Pvu II
Pst I
Pst I

FIG.5

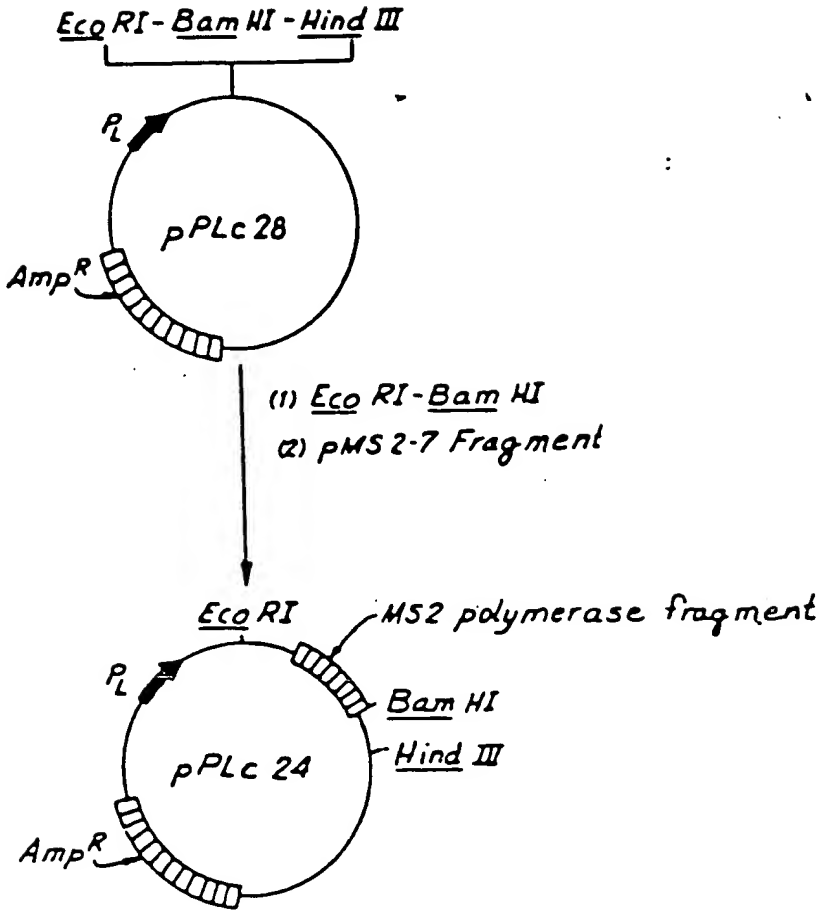
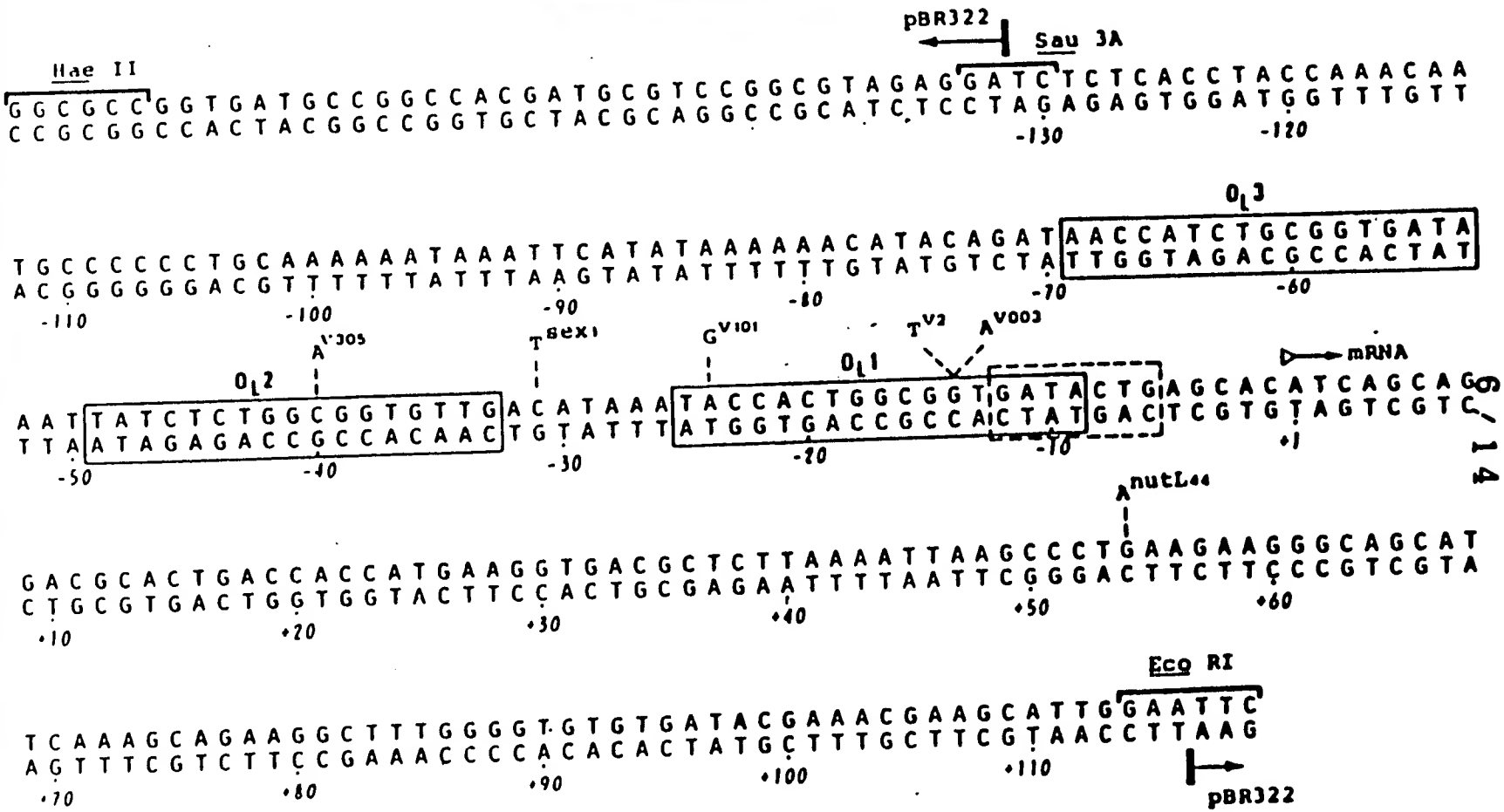

Eco RI - Bam HI - Hind III
P_L
pPLc 28
Amp^R
(1) Eco RI - Bam HI
(2) pMS 2-7 Fragment
Eco RI
MS2 polymerase fragment
P_L
Bam HI
Hind III
pPLc 24
Amp^R

FIG.6

LAMBDA PROMOTER P_L



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0041767

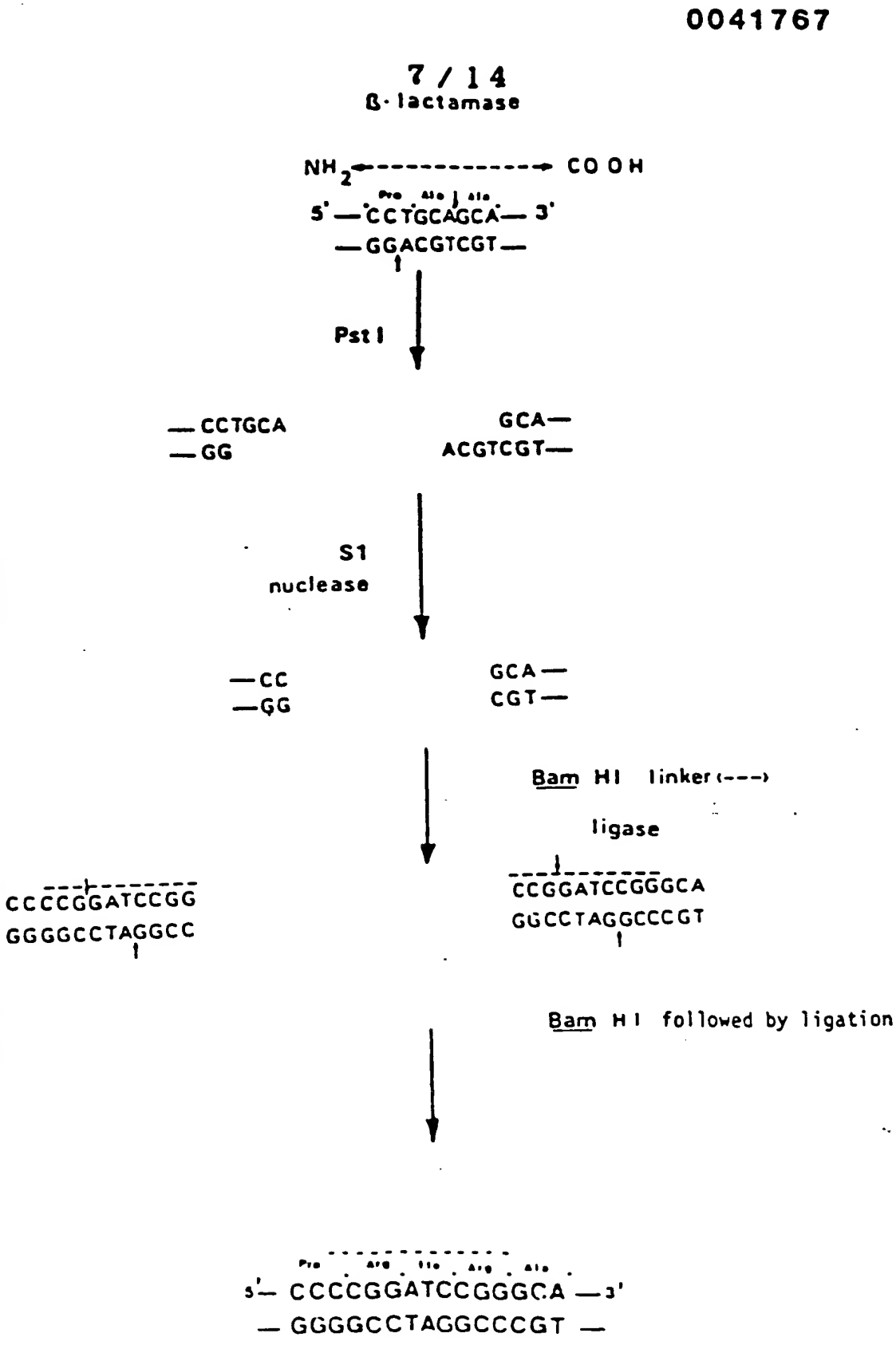
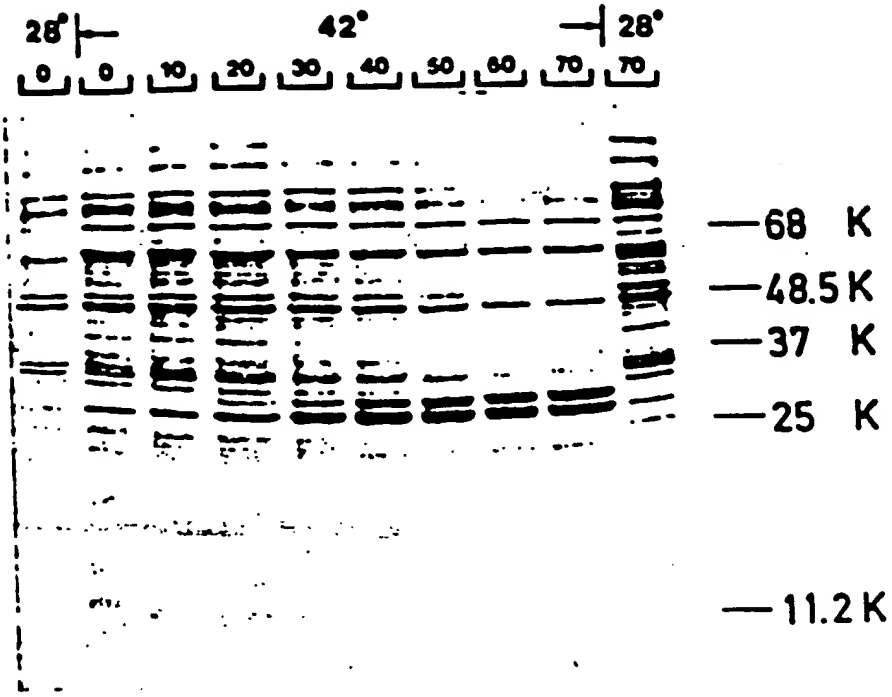
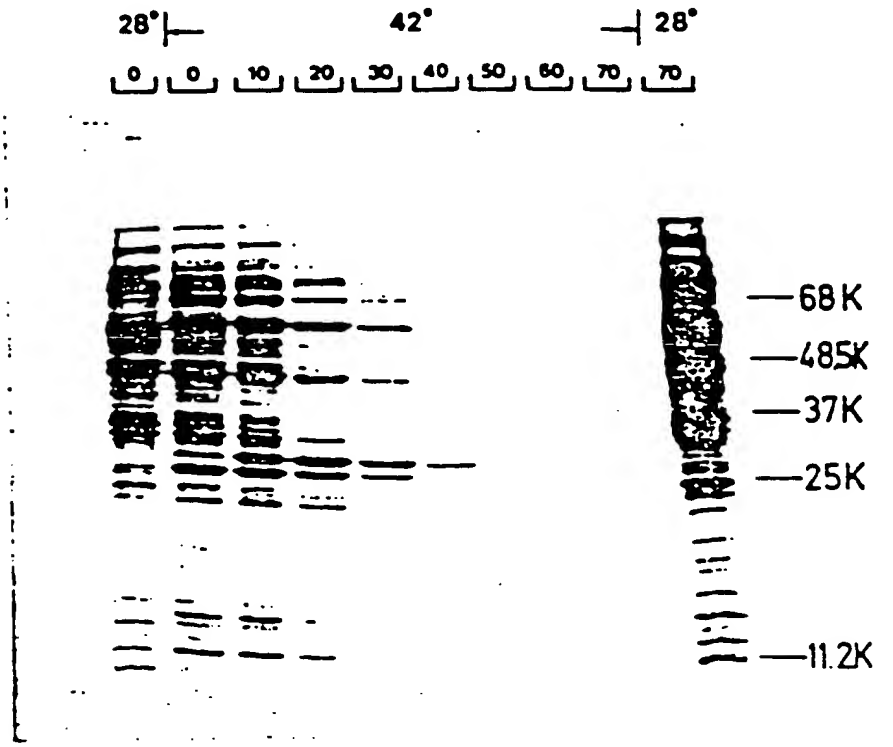

β-lactamase
NH2 ← → COOH
Pro Ala Ala
5' — CCTGCAGCA — 3'
— GGACGTCGT —
Pst I
— CCTGCA
— GG
GCA —
ACGTCGT —
S1
nuclease
— CC
— GG
GCA —
CGT —
Bam HI linker (---)
ligase
CCCCGGATCCGG
GGGGCCTAGGCC
CCGGATCCGGGCA
GGCCTAGGCCCGT
Bam HI followed by ligation
Pro Arg Ile Arg Ala
5' — CCCCGGATCCGGGCA — 3'
— GGGGCCTAGGCCCGT —

FIG.7

FIG.8

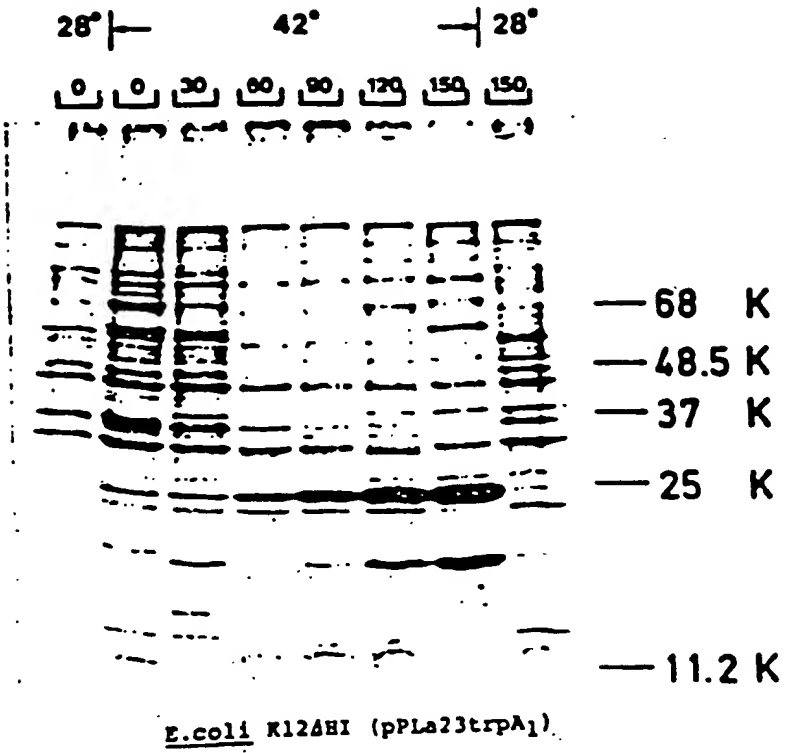


E.coli K12ΔHI (pPLa23)

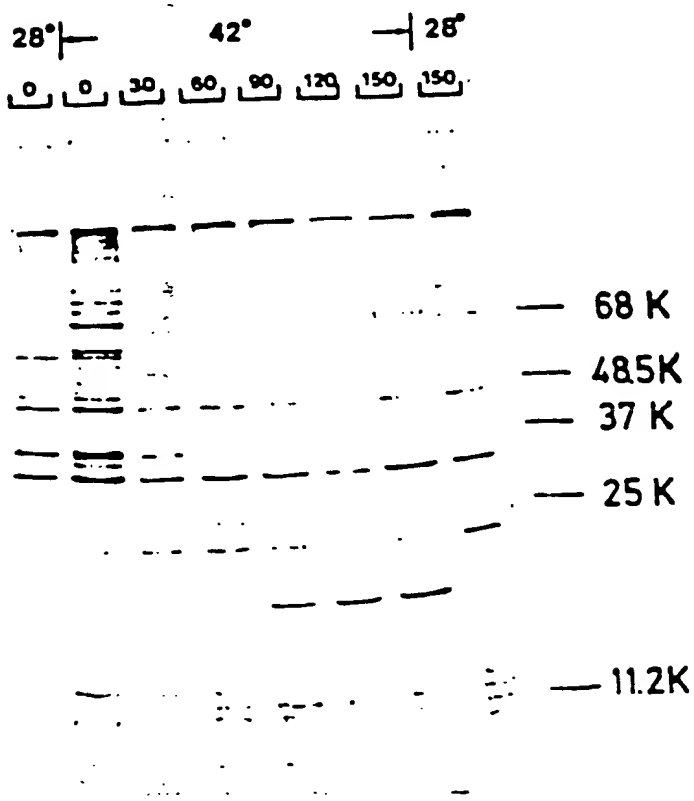


E.coli M5219 (pPLa23)

FIG.9

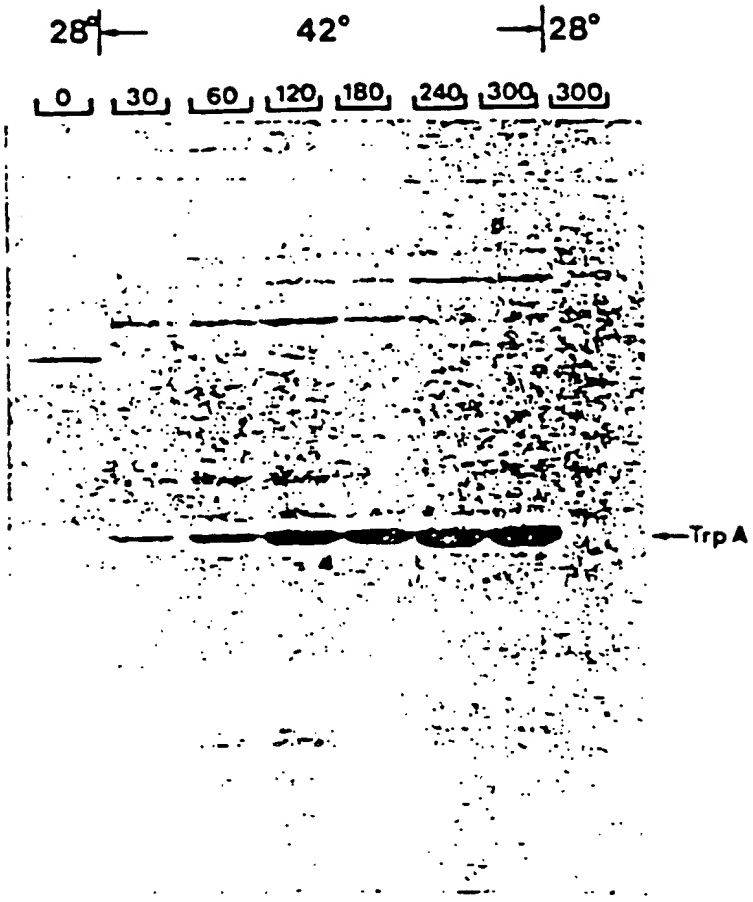


E.coli K12ΔHI (pPLa23trpA$_1$)



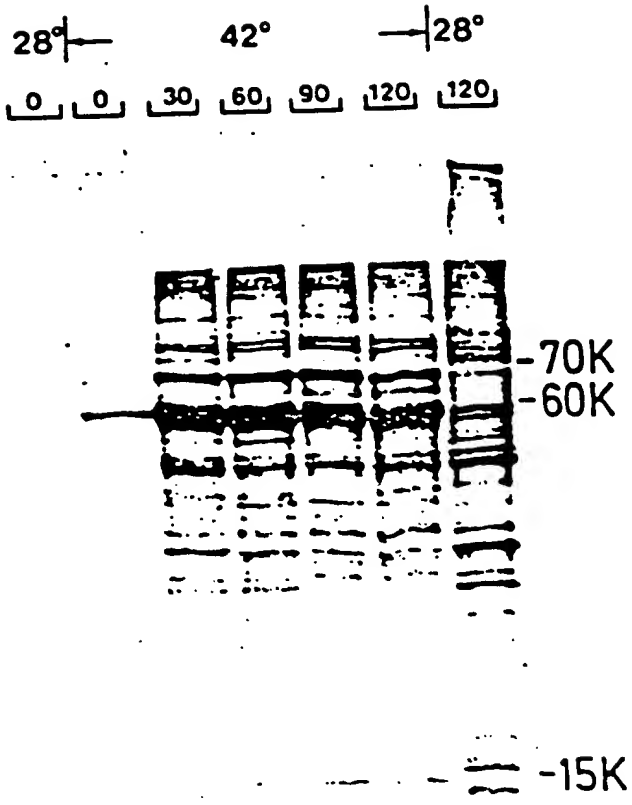
E.coli K12ΔHI (pPLa23trpA$_2$)

FIG. 10



E.coli K12ΔHI (pPLc23trpA$_1$)

FIG. II



E.coli K12ΔHI (pPLa2311R$_1$)

FIG. 12

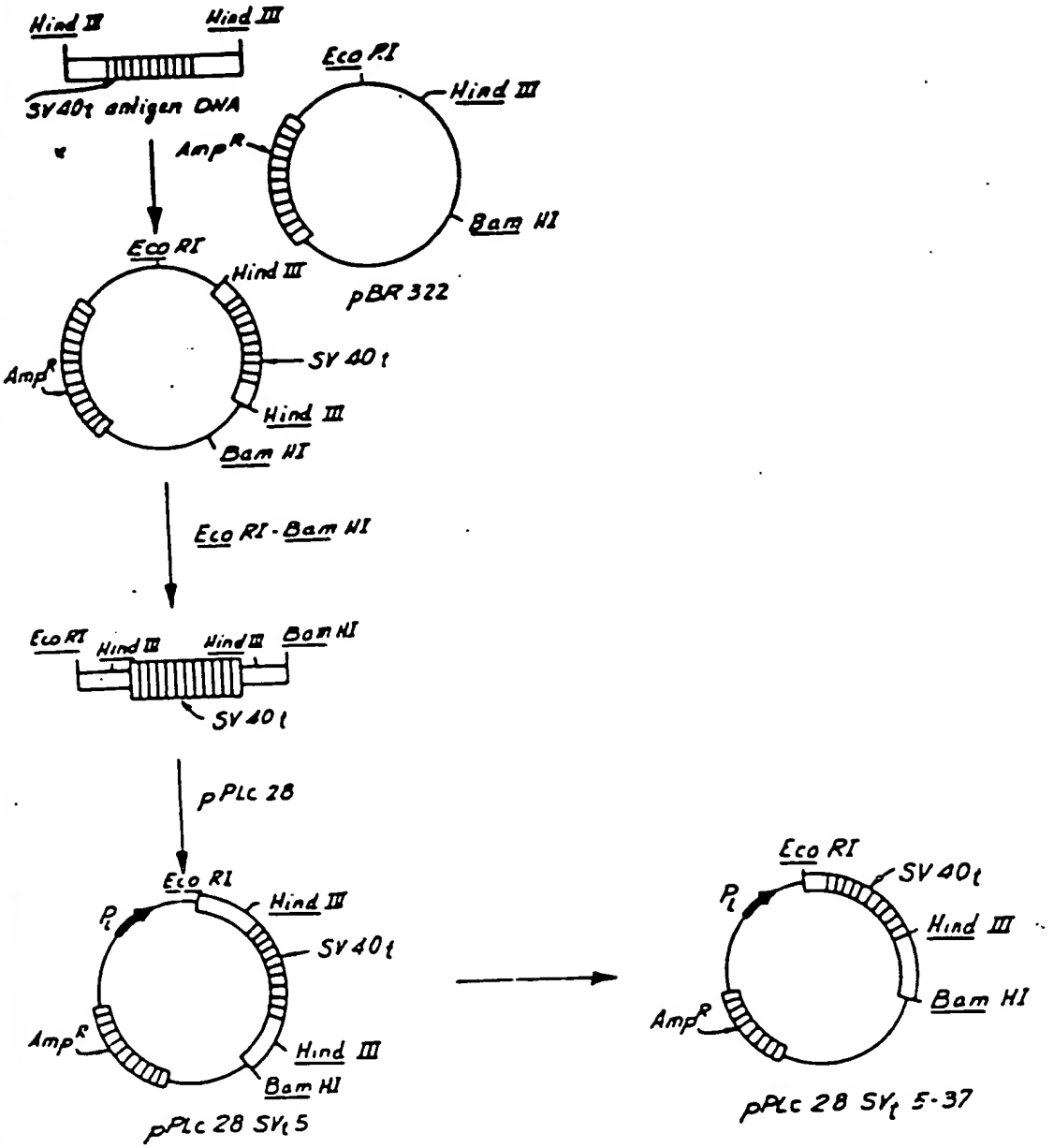

Hind III
Hind III
SV40t antigen DNA
Amp^R
Eco RI
Hind III
Bam HI
pBR 322
Eco RI
Hind III
SV 40 t
Amp^R
Hind III
Bam HI
Eco RI - Bam HI
Eco RI
Hind III
Hind III
Bam HI
SV 40 t
pPLc 28
Eco RI
Hind III
P_t
SV 40 t
Amp^R
Hind III
Bam HI
pPLc 28 SV_t 5
Eco RI
SV 40 t
P_t
Hind III
Bam HI
Amp^R
pPLc 28 SV_t 5-37

FIG. 13

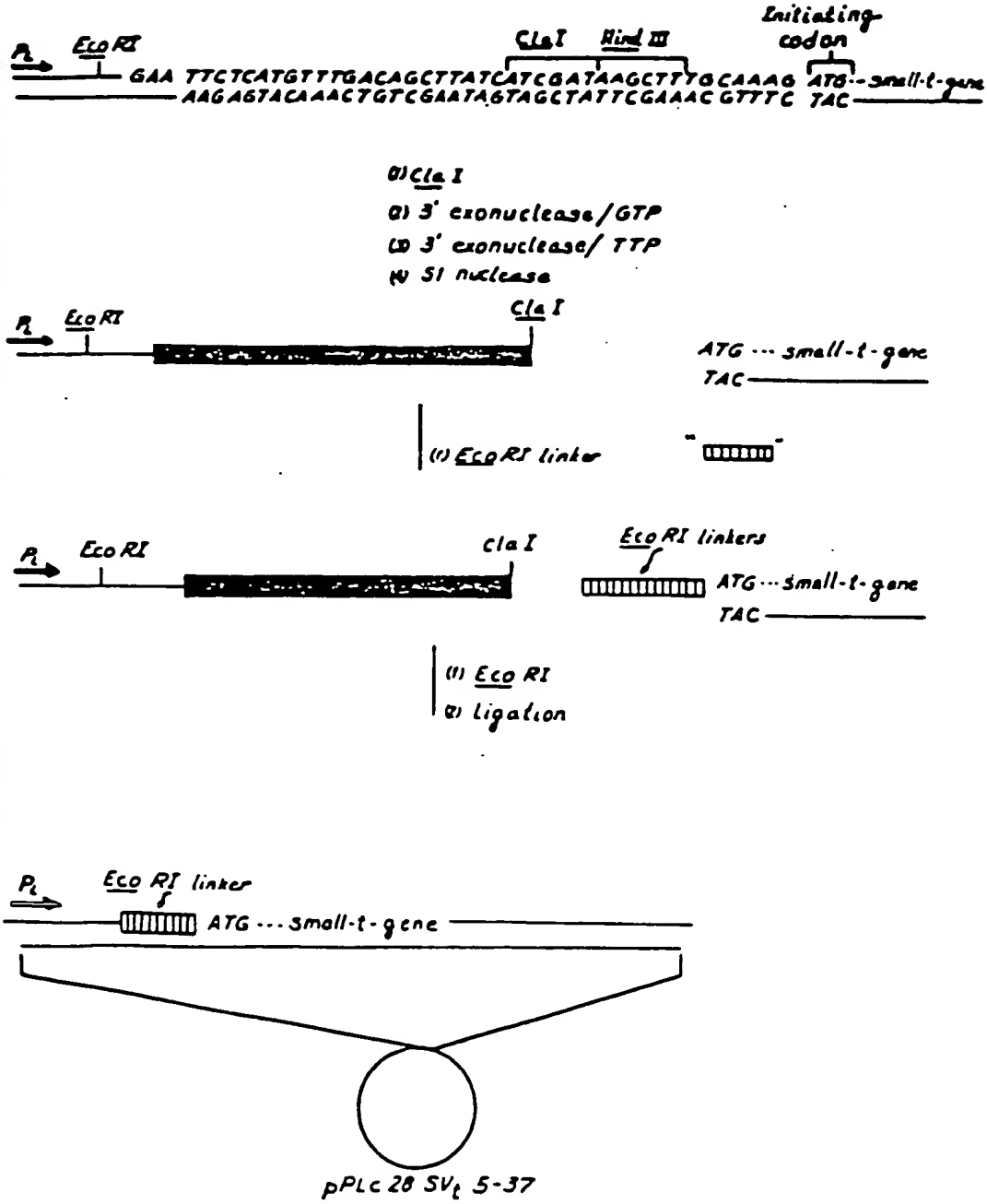
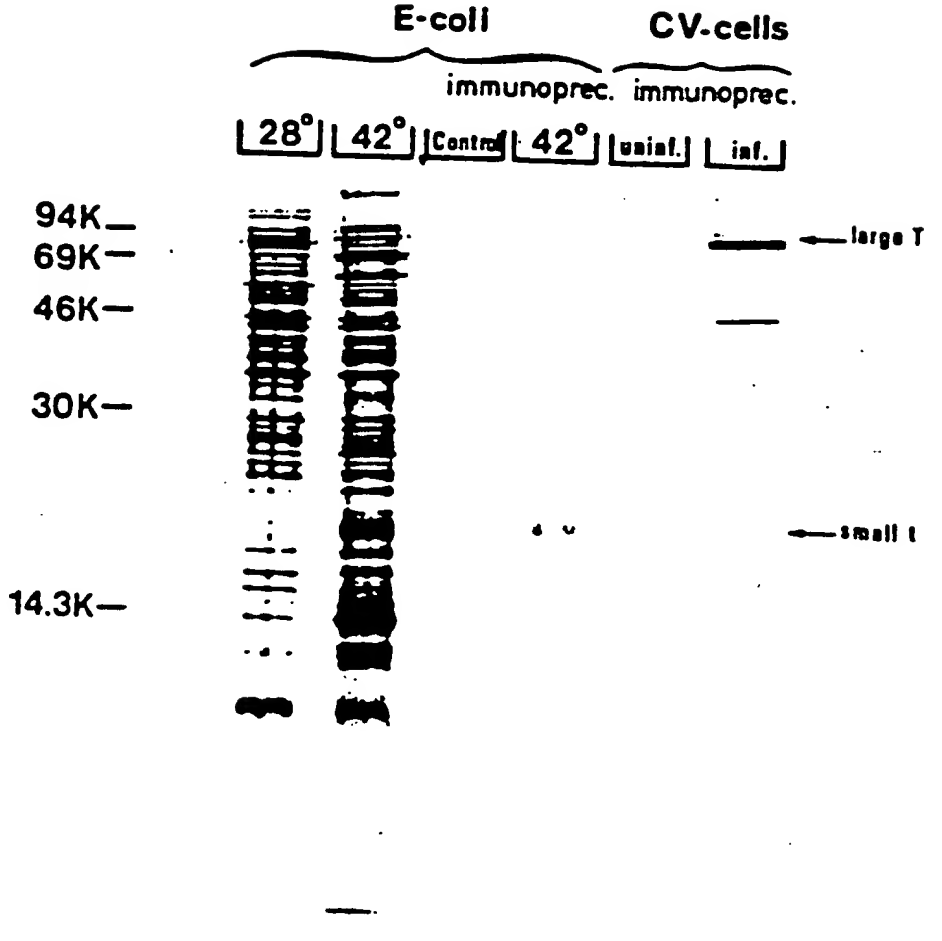

Initiating codon
ClaI
HindIII
EcoRI
GAA TTCTCATGTTTGACAGCTTATCATCGATAAGCTTTGCAAAG ATG--small-t-gene
AAGAGTACAAACTGTCGAATAGTAGCTATTCGAAACGTTTC TAC
a) ClaI
b) 3' exonuclease/GTP
c) 3' exonuclease/TTP
d) S1 nuclease
ATG ··· small-t-gene
TAC
e) EcoRI linker
EcoRI linkers
f) EcoRI
g) ligation
pPLc 28 SVt 5-37

FIG. 14



E.coli K12ΔHI (pPLc28SV$_{\epsilon}$5-37-9)